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(54) **Title:** METHOD FOR DETECTING HUMAN PAPILLOMARVIRUS MRNA

(57) **Abstract:** An in vitro method is provided for screening human female subjects to assess their risk of developing cervical carcinoma which comprises screening the subject for expression of mRNA transcripts from the E6 and optionally the L1 gene of human papillomavirus, wherein subjects positive for expression of L1 and/or E6 mRNA are scored as being at risk of developing cervical carcinoma. Kits for carrying out such methods are also provided.

METHOD FOR DETECTING HUMAN PAPILLOMAVIRUS mRNA

Field of the invention

The present invention relates to *in vitro* methods
of screening human subjects in order to assess their
risk of developing cervical carcinoma.

Background to the invention

Cervical carcinoma is one of the most common
malignant diseases world-wide and is one of the
leading causes of morbidity and mortality among women
(Parkin DM, Pisani P, Ferlay J (1993) Int J Cancer 54:
594-606;Pisani P, Parkin DM, Ferlay J (1993) Int J
Cancer 55: 891-903). 15,700 new cases of invasive
cervical cancer were predicted in the United States in
1996, and the annual world-wide incidence is estimated
to be 450,000 by the World Health Organization (1990).
The annual incidence rate differs in different parts
of the world, ranging from 7.6 per 100,000 in western
Asia to 46.8 per 100,000 in southern Africa (Parkin et
al., 1993 *ibid*).

The current conception of cervical carcinoma is
that it is a multistage disease, often developing over
a period of 10-25 years. Invasive squamous-cell
·carcinoma of the cervix is represented by penetration
through the basal lamina and invading the stroma or
epithelial lamina propria. The clinical course of
cervical carcinoma shows considerable variation.
Prognosis has been related to clinical stage, lymph
node involvement, primary tumour mass, histology type,
depth of invasion and lymphatic permeation (Delgado G,
et al., (1990) Gynecol Oncol 38: 352-357). Some
patients with less favourable tumour characteristics
have a relatively good outcome, while others suffer a
fatal outcome of an initially limited disease. This

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shows a clear need for additional markers to further
characterise newly diagnosed cervical carcinomas, in
order to administer risk-adapted therapy (Ikenberg H,
et al., Int. J. Cancer 59:322-6. 1994).

The epidemiology of cervical cancer has shown
strong association with religious, marital and sexual
patterns. Almost 100 case-control studies have
examined the relationship between HPV and cervical
neoplasia and almost all have found positive
associations (IARC monographs, 1995). The association
is strong, consistent and specific to a limited number
of viral types (Munoz N, Bosch FX (1992) HPV and
cervical neoplasia: review of case-control and cohort
studies. IARC Sci Publ 251-261). Among the most
informative studies, strong associations with HPV 16
DNA have been observed with remarkable consistency for
invasive cancer and high-grade CIN lesions, ruling out
the possibility that this association can be explained
by chance, bias or confounding (IARC monographs,
1995). Indirect evidence suggested that HPV DNA
detected in cancer cells is a good marker for the role
of HPV infection earlier in the carcinogenesis.
Dose-response relationship has been reported between
increasing viral load and risk of cervical carcinoma
(Munoz and Bosch, 1992 *ibid*). In some larger series
up to 100% of the tumours were positive for HPV but
the existence of virus-negative cervical carcinomas is
still debatable (Meijer CJ, et al., (1992) Detection
of human papillomavirus in cervical scrapes by the
polymerase chain reaction in relation to cytology:
possible implications for cervical cancer screening.
IARC Sci Publ 271-281; Das BC, et al., (1993) Cancer
72: 147-153).

The most frequent HPV types found in
squamous-cell cervical carcinomas are HPV 16 (41%-86%)

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and 18 (2%-22%). In addition HPV 31, 33, 35, 39, 45,
51, 52, 54, 56, 58, 59, 61, 66 and 68 are also found
(IARC, monographs, 1995). In the HPV2000 International
conference in Barcelona HPV 16, 18, 31 and 45 were
5   defined as high risk, while HPV 33, 35, 39, 51, 52,
56, 58, 59, 68 were defined as intermediate risk
(Keerti V. Shah. P71). The 13 high risk plus
intermediate risk HPVs are together often referred to
as cancer-associated HPV types.

10
     A number of studies have explored the potential
role of HPV testing in cervical screening (see Cuzick
et al. A systematic review of the role of human
papillomavirus testing withing a cervical screening
15   programme. Health Technol Assess 3:14. 1999).

     Reid et al., (Reid R, et al., (1991) Am J Obstet
Gynecol 164: 1461-1469) where the first to demonstrate
a role for HPV testing in a screening context. This
20   study was carried out on high-risk women from sexually
transmitted disease clinics and specialist
gynaecologists, and used a sensitive (low stringency)
Southern blot hybridisation for HPV detection. A
total of 1012 women were enrolled, and cervicography
25   was also considered as a possible adjunct to cytology.
Twenty-three CIN II/III lesions were found altogether,
but only 12 were detected by cytology (sensitivity
52%, specificity 92%). HPV testing found 16
high-grade lesions.

30
     Bauer et al. (Bauer HM, et al., (1991) JAMA 265:
472-477) report an early PCR-based study using MY09/11
primers (Manos M, et al.,(1990) Lancet 335: 734) in
young women attending for routine smears (college
35   students). They found a positive rate of 46% in 467
women, which was much higher than for dot blot assay
(11%).

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In a study using PCR with GP5/6 primers (Van Den
Brule AJ, et al., (1990) J Clin Microbiol 28:
2739-2743) van der Brule et al. (Van Den Brule AJ, et
al., (1991) Int J Cancer 48: 404-408) showed a very
strong correlation of HPV positivity with cervical
neoplasia as assessed by cytology. In older women
(aged 35-55 years) with negative cytology the HPV
positivity rate was only 3.5%, and this was reduced to
1.5% if only types 16, 18, 31 and 33 were considered,
while women with histological carcinoma in situ were
all HPV-positive, and 90% had one of the four above
types. Women with less severe cytological
abnormalities had lower HPV positivity rates in a
graded way, showing a clear trend.

Roda Housman et al. (Roda Housman AM, et al.,
(1994) Int J Cancer 56: 802-806) expanded these
observations by looking at a further 1373 women with
abnormal smears. This study also confirmed increasing
positivity rate with increasing severity of smear
results. They also noted that the level of HPV
heterogeneity decreased from 22 types for low-grade
smears to ten "high-risk" types for high grade smears.
This paper did not include any cytologically negative
women, nor was cytological disease confirmed
histologically.

Cuzick et al. (Cuzick J, et al., (1992) Lancet
340: 112-113; Cuzick J, et al., (1994) Br J Cancer 69:
167-171) were the first to report that HPV testing
provided useful information for the triage of
cytological abnormalities detected during random
screening. In a study of 133 women, referral for
coloposcopy they found a positive predictive value of
42%, which was similar to that for moderate
dyskaryosis. The results were most striking for HPV
16, where 39 of 42 HPV 16 positive women were found to

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have high-grade CIN on biopsy. This study pointed out the importance of assessing viral load and only considered high levels of high-risk types as positive.

5   Cox et al. (Cox JT, et al., (1995) Am J Obstet Gynecol 172: 946-954) demonstrated a role for HPV testing using the Hybrid Capture™ system (DIGENE Corporation, Gaithersburg, MD, USA) for triaging women with borderline smears. This test was performed on
10   217 such women from a college referral service, and a sensitivity of 93% was found for CINII/III compared with 73% for repeat cytology. High viral load was found to further improve performance by reducing false positives. When 5 RLU was taken as a cut-off, a PPV
15   of approximately 24% was found with no loss of sensitivity.

      Cuzick et al. (Cuzick J, et al., (1995) Lancet 345: 1533-1536) evaluated HPV testing in a primary
20   screening context in 1985 women attending for routine screening at a family planning clinic. Sensitivity using type-specific PCR for the four common HPV types (75%) exceeded that of cytology (46%), and the PPV for a positive HPV test (42%) was similar to that for
25   moderate dyskaryosis (43%).

      WO 91/08312 describes methods for determining the prognosis of individuals infected with HPV which comprise measuring the level of HPV activity by
30   detecting transcripts of all or a portion of the E6 and/or E7 HPV genes in a sample and comparing the measurements of HPV activity with a previously established relationship between activity and risk of progression to serious cervical dysplasia or
35   carcinoma.

      WO 99/29890 describes methods for the assessment

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of HPV infection based on the measurement and analysis of gene expression levels. In particular, WO 99/29890 describes methods which are based on measuring the levels of expression of two or more HPV genes (e.g. HPV E6, E7, L1 and E2) and then comparing the ratio of expression of combinations of these genes to provide an indication of the stage of HPV-based disease in a patient.

The present inventors have determined that it is possible to make a clinically useful assessment of HPV-associated disease based only on a simple positive/negative determination of expression of HPV L1 and E6 mRNA transcripts, with no requirement for accurate quantitative measurements of expression levels or for determination of differences in the levels of expression of the two transcripts. This method is technically simple and, in a preferred embodiment, is amenable to automation in a mid-to-high throughput format. Furthermore, on the basis of results obtained using the method of the invention the inventors have defined a novel scheme for classification of patients on the basis of risk of developing cervical carcinoma which is related to disease-relevant molecular changes in the pattern of HPV gene expression and is independent of CIN classification.

Therefore, in a first aspect the invention provides an *in vitro* method of screening human subjects to assess their risk of developing cervical carcinoma which comprises screening for expression of mRNA transcripts from the L1 gene and the E6 gene of human papillomavirus, wherein subjects positive for expression of L1 and/or full length E6 mRNA are scored as being at risk of developing cervical carcinoma.

A positive screening result in the method of the invention is indicated by positive expression of L1 mRNA and/or E6 mRNA in cells of the cervix. Positive expression of either one of these mRNAs or both mRNAs is taken as an indication that the subject is "at risk" for development of cervical carcinoma. Women who express E6 mRNA are at high risk of developing cell changes because oncogenic E6 and E7 bind to cell cycle regulatory proteins and act as a switch for cell proliferation. Clear expression of E6 mRNA provides a direct indication of cell changes in the cervix. Expression of L1 mRNA, with or without expression of E6 mRNA is also indicative of the presence of an active HPV.

In the wider context of cervical screening, women identified as positive for L1 and/or E6 mRNA expression may be selected for further investigation, for example using cytology. Thus, at one level the method of the invention may provide a technical simple means of pre-screening a population of women in order to identify HPV-positive subjects who may be selected for further investigation.

In a specific embodiment, the method of the invention may be used to classify subjects into four different classes of risk for developing cervical carcinoma on the basis of positive/negative scoring of expression of L1 and E6 mRNA.

Accordingly, in a further aspect the invention provides an *in vitro* method of screening human subjects to assess their risk of developing cervical carcinoma which comprises screening the subject for expression of mRNA transcripts of the L1 gene of HPV and mRNA transcripts of the E6 gene of HPV, and sorting the subject into one of four categories of

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risk for development of cervical carcinoma based on
expression of L1 and/or E6 mRNA according to the
following classification:

5   Risk category 1: subjects negative for expression of
     L1 mRNA but positive for expression of E6 mRNA from
     at least one of HPV types 16, 18, 31, 33, 35, 39, 45,
     52, 56, 58, 59, 66 or 68. Those individuals positive
     for expression of E6 mRNA from at least one of HPV
10   types 16, 18, 31 or 33 are scored as being at higher
     risk, for example in comparison to individuals
     negative for these types but positive for expression
     of E6 mRNA from at least one of HPV types 35, 39, 45,
     52, 56, 58, 59, 66 or 68.
15
     Risk category 2: subjects positive for expression of
     L1 mRNA and positive for expression of E6 mRNA from
     at least one of HPV types 16, 18, 31, 33, 35, 39, 45,
     52, 56, 58, 59, 66 or 68. Those individuals positive
20   for expression of E6 mRNA from at least one of HPV
     types 16, 18, 31 or 33 are scored as being at higher
     risk, for example in comparison to individuals
     negative for these types but positive for expression
     of E6 mRNA from at least one of HPV types 35, 39, 45,
25   52, 56, 58, 59, 66 or 68.

     Risk category 3: subjects positive for expression of
     L1 mRNA but negative for expression of E6 mRNA from
     the cancer-associated HPV types, (e.g. negative for
30   expression of E6 mRNA from HPV types 16, 18, 31, 33,
     35, 39, 45, 52, 56, 58, 59, 66 and 68).

     Risk category 4: subjects negative for expression of
     L1 mRNA and negative for expression of E6 mRNA.
35
         In a preferred embodiment, positive expression is
     indicated by the presence of more than 50 copies of

the transcript per ml (or total volume of the sample)
and negative expression is indicated by the presence
of less than 1 copy of the transcript per ml (or total
volume of the sample).

5

The above classification is based on molecular
events which are relevant to risk of developing
cervical carcinoma and is independent of the CIN
status of the subjects. Thus, this method of
10 classification may provide an alternative to the use
of cytology in the routine screening of women to
identify those at potential risk of developing
cervical carcinoma. The method may also be used as an
adjunct to cytology, for example as a confirmatory
15 test to confirm a risk assessment made on the basis of
cytology.

Women positive for expression of high risk E6
mRNA from one of HPV types 16, 18, 31 or 33 but
20 negative for expression of L1 are in the highest level
of risk of developing severe cell changes and cell
abnormalities. This is due to the fact that a
negative result for L1 mRNA expression is directly
indicative of integrated HPV, and therefore a higher
25 probability of high and constant expression of E6 and
E7. Integration of a virus in the human genome has
also a direct impact on the stability of the cells.
Integration of HPV also reduces the possibility of
regression of cell changes.

30

Women positive for expression of E6 mRNA from one
of HPV types 16, 18, 31 or 33 and positive for
expression of L1 mRNA have a "high risk" HPV
expression and it is still possible that the HPV has
35 been integrated. However, the risk of these women is
not classed as high as those who are L1 negative and
E6 positive, since there is a reasonable probability

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that they do not have integrated HPV.

Women negative for expression of E6 mRNA from HPV
types 16, 18, 31 or 33 but positive for expression of
5   E6 mRNA from another HPV type, e.g. 35, 39, 45, 52,
56, 58, 59, 66 and 68, are still considered "at risk"
and may therefore be placed in risk categories 1 or 2
(as defined above) depending on whether they are
positive or negative for expression of L1 mRNA.
10

Women positive for L1 mRNA but negative for E6
mRNA are scored as being at moderate risk. There may
be high-risk HPV types in the sample and L1 expression
is indicative of lytic activity. There may also be
15   integrated HPV types but only with viruses that are
rare. However, detection of lytic activity may show
that the cell may soon develop some changes.

In the wider context of cervical screening the
20   method of the invention may be used to classify women
according to risk of developing cervical carcinoma and
therefore provide a basis for decisions concerning
treatment and/or further screening. By way of
example: women in risk category 1, particularly those
25   who exhibit positive expression of E6 mRNA from at
least one of HPV types 16, 18, 31 or 33, might be
identified as requiring "immediate action", meaning
conisation or colposcopy, including a biopsy and
histology.
30

Women in risk category 2, as defined above, might
be scored as requiring immediate attention, meaning
colposcopy alone or colposcopy including a biopsy and
histology.
35

Women in risk category 3, as defined above, might
be scored as requiring immediate re-test, meaning

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recall for a further test for HPV expression immediately or after a relatively short interval, e.g. six months.

5   Women in risk category 4, as defined above, might be returned to the screening program, to be re-tested for HPV expression at a later date.

       In a further embodiment the invention provides an
10   in vitro method of screening human subjects for the presence of integrated HPV or a modified episomal HPV genome, which method comprises screening the subject for expression of mRNA transcripts from the L1 gene and the E6 gene of human papillomavirus, wherein
15   subjects negative for expression of L1 mRNA but positive for expression of E6 mRNA are scored as carrying integrated HPV.

       The term "integrated HPV" refers to an HPV genome
20   which is integrated into the human genome.

       The term "modified episomal HPV genome" is taken to mean an HPV genome which is retained within a cell of the human subject as an episome, i.e. not
25   integrated into the human genome, and which carries a modification as compared to the equivalent wild-type HPV genome, which modification leads to constitutive or persistent expression of transcripts of the E6 and/or E7 genes. The "modification" will typically be
30   a deletion, a multimerisation or concatermerisation of the episome, a re-arrangement of the episome etc affecting the regulation of E6/E7 expression.

       As aforesaid, the presence of integrated HPV or a
35   modified episomal HPV genome is indicated by a negative result for L1 mRNA expression, together with

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a positive result for expression of E6 mRNA in cells
of the cervix. Therefore, the ability to predict the
presence of integrated HPV or a modified episomal HPV
genome in this assay is critically dependent on the

5   ability to score a negative result for L1 mRNA
expression. This requires a detection technique which
has maximal sensitivity, yet produces minimal false-
negative results. In a preferred embodiment this is
achieved by using a sensitive amplification and real-

10   time detection technique to screen for the presence or
absence of L1 mRNA. The most preferred technique is
real-time NASBA amplification using molecular beacons
probes, as described by Leone et al., Nucleic Acids
Research., 1998, Vol 26, 2150-2155. Due to the

15   sensitivity of this technique the occurrence of false-
negative results is minimised and a result of
"negative L1 expression" can be scored with greater
confidence.

20   In a further embodiment, a method of screening
human subjects for the presence of integrated HPV or a
modified episomal HPV genome may be based on screening
for expression of E6 mRNA alone. Thus, the invention
relates to an *in vitro* method of screening human

25   subjects for the presence of integrated HPV or a
modified episomal HPV genome, which method comprises
screening the subject for expression of mRNA
transcripts from the E6 gene of human papillomavirus,
wherein subjects positive for expression of E6 mRNA

30   are scored as carrying integrated HPV or a modified
episomal HPV genome.

Moreover, individuals may be sorted into one of
two categories of risk for development of cervical

35   carcinoma based on an "on/off" determination of
expression of E6 mRNA alone. Therefore, the invention
provides an *in vitro* method of screening human

subjects to assess their risk of developing cervical carcinoma, which method comprises screening the subject for expression of mRNA transcripts of the E6 gene of HPV and sorting the subject into one of two categories of risk for development of cervical carcinoma based on expression of E6 mRNA, wherein individuals positive for expression of E6 mRNA are scored as carrying integrated HPV or a modified episomal HPV genome and are therefore classified as "high risk" for development of cervical carcinoma, whereas individuals negative for expression of E6 mRNA are scored as not carrying integrated HPV or a modified episomal HPV genome and are therefore classified as "no detectable risk" for development of cervical carcinoma.

Subjects are sorted into one of two categories of risk for development of cervical carcinoma based on an "on/off" determination of expression of E6 mRNA in cells of the cervix. Individuals positive for expression of E6 mRNA are scored as carrying integrated HPV or a modified episomal HPV genome and are therefore classified "high risk" for development of cervical carcinoma, whereas individuals negative for expression of E6 mRNA are scored as not carrying integrated HPV a modified episomal HPV genome and are therefore classified as "no detectable risk" for development of cervical carcinoma.

In the context of cervical screening classification of subjects into the two groups having "high risk" or "no detectable risk" for development of cervical carcinoma provides a basis for decisions concerning treatment and/or further screening. For example subjects in the high risk category may be scored as requiring immediate further analysis, e.g. by histological colposcopy, whilst those in the no

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detectable risk category may be referred back to the
screening program at three or five year intervals.
These methods are particularly useful for assessing
risk of developing carcinoma in subjects known to be
5   infected with HPV, e.g. those testing positive for HPV
DNA, or subjects who have previously manifested a
cervical abnormality via cytology or pap smear.
Subjects placed in the "no detectable risk" category
on the basis of E6 mRNA expression may have HPV DNA
10   present but the negative result for E6 expression
indicates that HPV is unrelated to oncogene activity
at the time of testing.

The presence of integrated HPV or a modified
15   episomal HPV genome, as indicated by a positive result
for E6 mRNA expression, is itself indicative that the
subject has abnormal cell changes in the cervix.
Therefore, the invention also relates to an *in vitro*
method of identifying human subjects having abnormal
20   cell changes in the cervix, which method comprises
screening the subject for expression of mRNA
transcripts of the E6 gene of HPV, wherein individuals
positive for expression of E6 mRNA are identified as
having abnormal cell changes in the cervix.
25

The term "abnormal cell changes in the cervix"
encompasses cell changes which are characteristic of
more severe disease than low-grade cervical lesions or
low squamous intraepithelial lesions, includes cell
30   changes which are characteristic of disease of equal
or greater severity than high-grade CIN (defined as a
neoplastic expansion of transformed cells), CIN
(cervical intraepithelial neoplasia) III, or high
squamous intraepithelial neoplasia (HSIL), including
35   lesions with multiploid DNA profile and "malignant"
CIN lesions with increased mean DNA-index values, high
percentage of DNA-aneuploidy and 2.5c Exceeding Rates

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(Hanselaar *et al.*, 1992, Anal Cell Pathol.,
4:315-324; Rihet *et al.*, 1996, J. Clin Pathol
49:892-896; and McDermott *et al.*, 1997, Br. J. Obstet
Gynaecol. 104:623-625).

Cervical Intraepithelial Neoplasia (abbreviated
"CIN"), also called Cervical Dysplasia, is a cervical
condition caused Human Papilloma Virus. CIN is
classified as I, II or III depending on its severity.
It is considered a pre-cancerous abnormality, but not
an actual cancer. The mildest form, CIN I, usually
goes away on its own, although rarely it can progress
to cancer. The more severe forms, CIN II and CIN III,
most often stay the same or get worse with time. They
can become a cancer, but almost never do if treated
adequately.

HPV has been identified as a causative agent in
development of cellular changes in the cervix, which
may lead to the development of cervical carcinoma.
These cellular changes are associated with
constitutive or persistent expression of E6/E7
proteins from the HPV viral genome. Thus, it is
possible to conclude that subjects in which expression
of E6 mRNA can be detected, particularly those
subjects who exhibit persistent E6 expression when
assessed over a period of time, already manifest
cellular changes in the cervix. These changes may
have taken place in only a very few cells of the
cervix, and may not be detectable by conventional
cytology. Nevertheless, with the use of sensitive,
specific and accurate methods for detection of E6 mRNA
it is possible to identify those subjects who already
exhibit cellular changes in the cervix at a much
earlier stage than would be possible using
conventional cytological screening. This will allow
earlier intervention with treatments aimed at

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preventing the development of cervical carcinoma.

As a result of HPV integration into the human
genome or as a result of the "modification" in a
modified episomal HPV genome, normal control of the
viral E6/E7 oncogene transcription is lost (Durst et
al., 1985, J Gen Virol, 66(Pt 7): 1515-1522; Pater and
Pater, 1985 Virology 145:313-318; Schwarz et al.,
1985, Nature 314: 111-114; Park et al., 1997, ibid).
In contrast, in premalignant lesions and HPV-infected
normal epithelium papillomaviruses predominate in
"unmodified" episomal forms, hence oncogene (E6/E7)
transcription may be absent or efficiently
down-regulated (Johnson et al., 1990, J Gen Virol,
71(Pt 7): 1473-1479; Falcinelli et al., 1993, J Med
Virol, 40: 261-265). Integration of human
papillomavirus type 16 DNA into the human genome is
observed to lead to a more unstable cell
activity/genome, and increased stability of E6 and E7
mRNAs (Jeon and Lambert, 1995, Proc Natl Acad Sci USA
92: 1654-1658). Thus HPV integration, typically found
in cervical cancers but only infrequently found in CIN
lesions (Carmody et al., 1996, Mol Cell Probes, 10:
107-116), appears to be an important event in cervical
carcinogenesis.

The present methods detect E6/E7 viral mRNA
expression in the cervix instead of DNA. E6/E7 viral
expression in cervical cells is a much more accurate
assessment of the risk of developing cancer than
simply showing that the HPV virus is present.
Furthermore, the detection of HPV oncogene transcripts
may be a more sensitive indicator of the direct
involvement of viral oncogenes in carcinogenesis (Rose
et al., 1994, Gynecol Oncol, 52: 212-217; Rose et al.,
1995, Gynecol Oncol, 56: 239-244). Detection of E6/E7

transcripts by amplification and detection is a useful
diagnostic tool for risk evaluations regarding the
development of CIN and its progression to cervical
cancer, especially in high-risk HPV type-infected
5   patients with ASCUS and CIN I (Sotlar et al., 1998,
Gynecol Oncol, 69: 114-121; Selinka et al., 1998, Lab
Invest, 78: 9-18).

The expression of E6/E7 transcripts of HPV-16/18
10   is uniformly correlated with the physical status of
HPV DNAs (Park et al., 1997, Gynecol Oncol, Vol:65(1),
121-9). In most cervical carcinoma cells the E6 and
E7 genes of specific human papillomaviruses are
transcribed from viral sequences integrated into host
15   cell chromosomes (von Kleben Doeberitz et al., 1991,
Proc Natl Acad Sci U S A. Vol:88(4), 1411-5). Viral
load and integration has been evaluated in a large
series of CIN lesions (Pietsaro et al., 2002, J Clin
Microbiol, Vol:40(3), 886-91). Only one sample
20   contained exclusively episomal HPV16 DNA, and this
lesion regressed spontaneously. Seventeen of 37
invasive cervical carcinoma samples were identified
previously as containing the completely integrated
HPV16 genome by using PCR covering the entire E1/E2
25   gene, and this was confirmed by rliPCR in 16 cases.
·One case, however, showed a low level of episomal
deoxyribonucleic acid in addition to the predominant
integrated form. Of the remaining 20 carcinoma
samples showing episomal forms in the previous
30   analysis, 14 were found to contain integrated forms
using rliPCR, and four contained multimeric (modified)
episomal forms. Thus, in total, 31 of 37 of the
carcinomas (84%) showed integrated HPV16 genome, while
absence of integration could not be detected.
35   (Kalantari et al., 2001, Diagn Mol Pathol, Vol:10(1),
46-54).

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There have been virtually no observations that cervical carcinoma cells exist without integrated HPV or modified episomal HPV DNA (Kalantari et al. 2001; Pietsaro et al., 2002, *ibid*). It has further been shown that E6 and E7 may only be transcribed from integrated or modified episomal HPV DNA (von Kleben Doeberitz *et al.*, 1991, *ibid*). Therefore, the inventors surmise that detection of E6/E7 expression provides a direct indication of integrated HPV or modified episomal HPV and high oncogene activity, and conclude that in a clinical context detection of E6 (E6/E7) expression alone is sufficient to identify subjects at "high risk" of developing cervical carcinoma. In other words, if E6/E7 mRNA expression can be detected in a cervical sample, this is directly indicative of cellular abnormalities in the cervix and there is a very high risk of development of cervical carcinoma due to persistent HPV oncogene activity. Therefore, detection of E6/E7 mRNA in a human subject indicates that the subject has a very high risk of developing cervical carcinoma ·and should undergo immediate further screening, e.g. by colposcopy.

If HPV E6/E7 mRNA expression is not detected, the subject may still have an HPV infection. However due ·to absence of integration and oncogene activity, it may regress spontaneously (as observed by Pietsaro *et al.*, 2002, *ibid*).

In a clinical context the performance of methods which rely on screening for expression of E6 mRNA alone is critically dependent on the ability to score a negative result for E6 mRNA expression with confidence. This again requires a detection technique which has maximal sensitivity, yet produces minimal false-negative results. In a preferred embodiment

this is achieved by using a sensitive amplification
and real-time detection technique to screen for the
presence or absence of E6 mRNA. The most preferred
technique is real-time NASBA amplification using
5   molecular beacons probes, as described by Leone et
al., Nucleic Acids Research., 1998, Vol 26, 2150-2155.
Due to the sensitivity of this technique the
occurrence of false-negative results is minimised and
a result of "negative E6 expression" can be scored
10   with greater confidence. This is extremely important
if the assays are to be used in the context of a
clinical screening program.

In the methods based on detection of E6 mRNA
15   alone it is preferred to detect at least types HPV 16,
18, 31, 33 and 45, and in a preferred embodiment the
assay may detect only these HPV types. DNA from HPV
types 16, 18, 31 and 33 has been detected in more than
87% of cervical carcinoma samples (Karlsen et al.,
20   1996, J Clin Microbiol, 34:2095-2100). Other studies
have shown that E6 and E7 are almost invariably
retained in cervical cancers, as their expression is
likely to be necessary for conversion to and
maintenance of the malignant state (Choo et al., 1987,
25   J Med Virol 21:101-107; Durst et al., 1995, Cancer
Genet Cytogenet, 85: 105-112). In contrast to HPV
detection systems which are based on detection of the
undamaged genome or the L1 gene sequence, detection of
HPV mRNA expressed from the E6/E7 area may detect more
30   than 90% of the patients directly related to a risk of
developing cervical carcinoma.

In the clinic, methods based on detection of E6
mRNA are preferred for use in post-screening, i.e.
35   further analysis of individuals having a previous
diagnosis of ASCUS, CIN 1 or Condyloma. The method
may be used to select those with a high risk of

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developing cervical carcinoma from amongst the group
of individuals having a previous diagnosis of ASCUS,
CIN 1 or Condyloma. ASCUS, Condyloma and CIN I may be
defined as more or less the same diagnosis due to very
5   low reproducibility between different cytologists and
different cytological departments. Östör (Int J. Gyn
Path. 12:186-192. 1993) found that only around 1% of
the CIN 1 cases may progress to cervical carcinoma.
Thus, there is a genuine need for an efficient method
10 of identifying the subset of individuals with ASCUS,
Condyloma or CIN I who are at substantial risk of
developing cervical carcinoma. One of HPV types 16,
18, 31 or 33 was detected in 87% of the cervical
carcinoma cases study by Karlsen et al., 1996. By
15 inclusion of HPV 45, nearly 90% of the cervical
carcinoma samples are found to be related to these
five HPV types. Therefore, calculated from the data
provided by Östör (Int J. Gyn Path. 12:186-192. 1993)
more than 99.9% are detected cases with ASCUS, CIN I
20 or condyloma are missed by our HPV-Proofer kit.

In the methods of the invention "positive
expression" of an mRNA is taken to mean expression
above background. There is no absolute requirement
25 for accurate quantitative determination of the level
of mRNA expression or for accurate determination of
the relative levels of expression of L1 and E6 mRNA.

In certain embodiments, the methods of the
30 invention may comprise a quantitative determination of
levels of mRNA expression. In a preferred embodiment
in order to provide a clear distinction between
"positive expression" and "negative expression" a
determination of "positive expression" may require the
35 presence of more than 50 copies of the relevant mRNA
(per ml of sample or per total volume of sample),
whereas a determination of "negative expression" may

require the presence of less than 1 copy of the
relevant mRNA (per ml of sample or per total volume of
sample).

5   The methods of the invention will preferably
involve screening for E6 mRNA using a technique which
is able to detect specifically E6 mRNA from cancer-
associated HPV types, more preferably "high risk"
cancer-associated HPV types. In the most preferred
10   embodiment the methods involve screening for E6 mRNA
using a technique which is able to detect E6 mRNA from
HPV types 16, 18, 31 and 33, and preferably also 45.
Most preferably, the method will specifically detect
expression of E6 mRNA from at least one of HPV types
15   16, 18, 31, 33, and preferably also 45, and most
preferably all five types. However, women positive
for positive for expression of E6 from other types
than 16, 18, 31, 33 and 45, e.g. 35, 39, 45, 52, 56,
58, 59, 66 and 68 may still be "at risk" of developing
20   cervical carcinoma. Thus, the method may encompass
screening for expression of E6 mRNA from one or more
of these HPV types, most preferably in addition to
screening for E6 mRNA from HPV types 16, 18, 31, 33
and 45. Certain HPV types exhibit a marked
25   geographical/population distribution. Therefore, it
may be appropriate to include primers specific for an
HPV type known to be prevalent in the
population/geographical area under test, for example
in addition to screening for HPV types 16, 18, 31, 33
30   and 45.

For the avoidance of doubt, unless otherwise
stated the term "E6 mRNA" as used herein encompasses
all naturally occurring mRNA transcripts which contain
35   all or part of the E6 open reading frame, including
naturally occurring splice variants, and therefore
includes transcripts which additionally contain all or

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part of the E7 open reading frame (and indeed further
open reading frames). The terms "E6/E7 mRNA", "E6/E7
transcripts" etc are used interchangeably with the
terms "E6 mRNA", "E6 transcripts" and also encompass
5   naturally occuring mRNA transcripts which contain all
or part of the E6 open reading frame, including
naturally occurring splice variants, and transcripts
which contain all or part of the E7 open reading
frame. The term "oncogene expression", unless
10   otherwise stated, also refers to naturally occuring
mRNA transcripts which contain all or part of the E6
open reading frame, including naturally occurring
splice variants, and transcripts which contain all or
part of the E7 open reading frame.
15

Four E6/E7 mRNA species have so far been
described in cells infected with HPV 16, namely an
unspliced E6 transcript and three spliced transcripts
denoted E6*I, E6*II and E6*III (Smotkin D, et al., J
20   Virol. 1989 Mar 63(3):1441-7; Smotkin D, Wettstein FO.
Proc Natl Acad Sci USA. 1986 Jul 83(13):4680-4;
Doorbar J. et al., Virology. 1990 Sep 178(1):254-62;
Cornelissen MT, et al. J Gen Virol. 1990 May 71(Pt
5):1243-6; Johnson MA, et al. J Gen Virol. 1990 Jul
25   71(Pt 7):1473-9; Schneider-Maunoury S, et al. J Virol.
1987 Oct 61(10):3295-8; Sherman L, et al. Int J
Cancer. 1992 Feb 50(3):356-64). All four transcripts
are transcribed from a single promoter (p97) located
just upstream of the second ATG of the E6 ORF.
30

In one embodiment the methods may comprise
screening for E6 transcripts which contain all or part
of the E7 open reading frame, This may be
accomplished, for example, using primers or probes
35   specific for the E7 coding region.

In a further embodiment, the methods may comprise

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screening for the presence of "full length" E6
transcripts. In the case of HPV 16 the term "full
length E6 transcripts" refers to transcripts which
contain all of the region from nucleotide (nt) 97 to
5   nt 880 in the E6 ORF, inclusive of nt 97 and 880.
Nucleotide positions are numbered according to
standard HPV nomenclature (see Human Papillomavirus
Compendium OnLine, available via the internet or in
paper form from HV Database, Mail Stop K710, Los
10   Alamos National Laboratory, Los Alamos, NM 87545,
USA). Specific detection of full length transcripts
may be accomplished, for example, using primers or
probes which are specific for the region which is
present only in full length E6 transcripts, not in
15   splice variants. Different HPV types exhibit
different patterns of E6/E7 mRNA expression.
Transcript maps for various HPV types, including HPV
types 16 and 31, which may be used to assist in the
design of probes or primers for detection of E6/E7
20   transcripts are publicly available via the Human
Papillomavirus Compendium (as above).

       E6 oligonucleotide primers are described herein
which are suitable for use in amplification of regions
25   of the E6 mRNA from various HPV types by NASBA or PCR.

       In a preferred embodiment methods which involve
screening for L1 mRNA expression may comprise
screening for L1 mRNA expression using a technique
30   which is able to detect L1 mRNA from substantially all
known HPV types or at least the major cancer-
associated HPV types (e.g. preferably all of HPV types
16, 18, 31 and 33) . L1 primers and probes are
described herein which are capable of detecting L1
35   mRNA from HPV types 6, 11, 16, 18, 31, 33, 35 and 51
in cervical samples.

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Detection of L1 transcripts can be said to detect
HPV "virulence", meaning the presence of HPV lytic
activity. Detection of E6/E7 transcripts can be said
to detect HPV "pathogenesis" since expression of these
5   mRNAs is indicative of molecular events associated
with risk of developing carcinoma.

In a study of 4589 women it was possible to
detect all except one case of CIN III lesions or
10   cancer using a method based on screening for
expression of E6 and L1 mRNA (see accompanying
Examples).

In further embodiments, the above-described
15   methods of the invention may comprise screening for
expression of mRNA transcripts from the human p16^{ink4a}
gene, in addition to screening for expression of HPV
L1 and/or E6 transcripts.

20   A positive result for expression of p16^{ink4a} mRNA
is taken as a further indication of risk of developing
cervical carcinoma.

P16^{ink4a}, and the related family members, may
25   function to regulate the phosphorylation and the
growth suppressive activity of the restinoblastoma
gene product (RB). In support of this, it has been
found that there is an inverse relationship between
the expression of p16^{ink4a} protein and the presence of
30   normal RB in selected cancer cell lines; p16^{ink4a}
protein is detectable when RB is mutant, deleted, or
inactivated, and it is markedly reduced or absent in
cell lines that contain a normal RB. Kheif et
al.(Kheif SN et al., Proc. Natl. Acad. Sci. USA
35   93:4350-4354. 1996), found that p16^{ink4a} protein is
expressed in human cervical carcinoma cells that
contain either a mutant RB or a wild-type RB that is

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functionally inactivated by E7. They also show that
the inactivation of RB correlates with an upregulation
of p16^{ink4a} confirming a feedback loop involving p16^{ink4a}
and RB. Milde-Langosch et al. (Milde-Langosch K, et
5   al., (2001) Virchows Arch 439: 55-61) found that there
were significant correlations between strong p16
expression and HPV16/18 infection and between strong
p16 expression and HPV 16/18 E6/E7 oncogene
expression. Klaes et al., (Klaes R, et al., (2001) Int
10   J Cancer 92: 276-284) observed a strong over
expression of the p16^{ink4a} gene product in 150 of 152
high-grade dysplastic cervical lesions (CIN II to
invasive cancer), whereas normal cervical epithelium
or inflammatory or metaplastic lesions were not
15   stained with the p16^{ink4a} specific monoclonal antibody
E6H4. All CIN I scored lesions associated with LR-HPV
types displayed no or only focal or sporadic
reactivity, whereas all but two CIN I scored lesions
associated with HR-HPV types showed strong and diffuse
20   staining for p16^{ink4a}.

The disclosed screening methods may be carried
out on a preparation of nucleic acid isolated from a
clinical sample or biopsy containing cervical cells
25   taken from the subject under test. Suitable samples
which may be used as a source of nucleic acid include
(but not exclusively) cervical swabs, cervical
biopsies, cervical scrapings, skin biopsies/warts,
also paraffin embedded tissues, and formalin or
30   methanol fixed cells.

The preparation of nucleic acid to be screened
using the disclosed method must include mRNA, however
it need not be a preparation of purified poly A+ mRNA
35   and preparations of total RNA or crude preparations of
total nucleic acid containing both RNA and genomic
DNA, or even crude cell lysates are also suitable as

starting material for a NASBA reaction. Essentially any technique known in the art for the isolation of a preparation of nucleic acid including mRNA may be used to isolate nucleic acid from a test sample. A

5   preferred technique is the "Boom" isolation method described in US-A-5,234,809 and EP-B-0389,063. This method, which can be used to isolate a nucleic acid preparation containing both RNA and DNA, is based on the nucleic acid binding properties of silicon dioxide

10   particles in the presence of the chaotropic agent guanidine thiocyanate (GuSCN).

The methods of the invention are based on assessment of active transcription of the HPV genome

15   in cervical cells. The methods are not limited with respect to the precise technique used to detect mRNA expression. Many techniques for detection of specific mRNA sequences are known in the art and may be used in accordance with the invention. For example, specific

20   mRNAs may be detected by hybridisation, amplification or sequencing techniques.

It is most preferred to detect mRNA expression by means of an amplification technique, most preferably

25   an isothermal amplification such as NASBA, transcription-mediated amplification, signal-mediated amplification of RNA technology, isothermal solution phase amplification, etc. All of these methods are well known in the art More preferably mRNA expression

30   is detected by an isothermal amplification in combination with real-time detection of the amplification product. The most preferred combination is amplification by NASBA, coupled with real-time detection of the amplification product using molecular

35   beacons technology, as described by Leone et al., Nucleic Acids Research, 1998, Vol 26, 2150-2155.

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Methods for the detection of HPV in a test sample using the NASBA technique will generally comprise the following steps:

(a) assembling a reaction medium comprising
5   suitable primer-pairs, an RNA directed DNA polymerase, a ribonuclease that hydrolyses the RNA strand of an RNA-DNA hybrid without hydrolysing single or double stranded RNA or DNA, an RNA polymerase that recognises said promoter, and ribonucleoside and
10   deoxyribonucleoside triphosphates;

(b) incubating the reaction medium with a preparation of nucleic acid isolated from a test sample suspected of containing HPV under reaction conditions which permit a NASBA amplification
15   reaction; and

(c) detecting and/or quantitatively measuring any HPV-specific product of the NASBA amplification reaction.

20   Detection of the specific product(s) of the NASBA reaction (i.e. sense and/or antisense copies of the target RNA) may be carried out in a number of different ways. In one approach the NASBA product(s) may be detected with the use of an HPV-specific
25   hybridisation probe capable of specifically annealing to the NASBA product. The hybridisation probe may be attached to a revealing label, for example a fluorescent, luminescent, radioactive or chemiluminescent compound or an enzyme label or any
30   other type of label known to those of ordinary skill in the art. The precise nature of the label is not critical, but it should be capable of producing a signal detectable by external means, either by itself or in conjunction with one or more additional
35   substances (e.g. the substrate for an enzyme).

A preferred detection method is so-called "real-

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time NASBA" which allows continuous monitoring of the
formation of the product of the NASBA reaction over
the course of the reaction. In a preferred embodiment
this may be achieved using a "molecular beacons" probe
5   comprising an HPV-specific sequence capable of
annealing to the NASBA product, a stem-duplex forming
oligonucleotide sequence and a pair of
fluorescer/quencher moieties, as known in the art and
described herein. If the molecular beacons probe is
10   added to the reaction mixture prior to amplification
it may be possible to monitor the formation of the
NASBA product in real-time (Leone *et al.*, Nucleic
Acids Research, 1998, Vol 26, 2150-2155). Reagent
kits and instrumentation for performing real-time
15   NASBA detection are available commercially (e.g.
NucliSens™ EasyQ system, from Organon Teknika).

In a further approach, the molecular beacons
technology may be incorporated into the primer 2
20   oligonucleotide allowing real-time monitoring of the
NASBA reaction without the need for a separate
hybridisation probe.

In a still further approach the products of the
25   NASBA reaction may be monitored using a generic
labelled detection probe which hybridises to a
nucleotide sequence in the 5' terminus of the primer 2
oligonucleotide.   This is equivalent to the
"NucliSens™" detection system supplied by Organon
30   Teknika. In this system specificity for NASBA
products derived from the target HPV mRNA may be
conferred by using HPV-specific capture probes
comprising probe oligonucleotides as described herein
attached to a solid support such as a magnetic
35   microbead. Most preferably the generic labelled
detection probe is the ECL™ detection probe supplied
by Organon Teknika. NASBA amplicons are hybridized to

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the HPV-specific capture probes and the generic ECL
probe (via a complementary sequence on primer 2).
Following hybridization the bead/amplicon/ECL probe
complexes may be captured at the magnet electrode of
5   an automatic ECL reader (e.g. the NucliSens™ reader
supplied by Organon Teknika). Subsequently, a voltage
pulse triggers the ECL™ reaction.

The detection of HPV mRNA is also of clinical
10   relevance in cancers other than cervical carcinoma
including, for example, head and neck carcinoma, oral
and tongue carcinoma, skin carcinoma, anal and vaginal
carcinoma. Detection of HPV mRNA may also be very
useful in the diagnosis of micrometastases in lymph
15   nodes in the lower part of the body. Hence, the
invention also contemplates screens for susceptibility
to the above-listed cancers based on screening for
expression of HPV L1 and E6 transcripts.

20   In accordance with a further aspect of the
invention there is provided a kit for use in the
detection of transcripts of the L1 and E6 genes of
HPV, the kit comprising at least one primer-pair
suitable for use in amplification of a region of L1
25   transcripts from at least HPV types 16, 18, 31 and 33,
and preferably also HPV 45, and one or more primer-
pairs which enable amplification of a region of E6
transcripts from HPV types 16, 18, 31 and 33, and
preferably also HPV 45.
30
"Primer-pair" taken to mean are pair of primers
which may be used in combination to amplify a specific
region of the L1 or E6 mRNA using any known nucleic
acid technique. In preferred embodiments the primer-
35   pairs included in the kit will be suitable for use in
NASBA amplification or similar isothermal
amplification techniques.

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The individual primers making up each primer-pair
included in the kit may be supplied separately (e.g. a
separate container of each primer) or, more
preferably, may be supplied mixed in a single
5   container. Combinations of two or more primer-pairs
may be supplied ready-mixed in a single container
within the kit. It may be convenient to supply two or
more primer-pairs in a single container where the two
or more amplification reactions are to be
10   "multiplexed", meaning performed simultaneously in a
single reaction vessel.

The primer-pair(s) suitable for use in
amplification of a region of E6 transcripts should
15   enable amplification a region of E6 mRNA from at least
the major cancer-associated HPV types 16, 18, 31 and
33, and preferably also HPV 45. There are several
different ways in which this can be achieved.

20   In one embodiment, the kit may contain separate
primer-pairs specific for each of HPV types 16, 18, 31
and 33, and preferably also HPV 45. These primer-
pairs may be supplied within the kit in separate
containers, or they may be supplied as mixtures of two
25   or more primer-pairs in a single container, for
.example to enable multiplexing of the amplification
reactions.

In a further embodiment, the kit may contain a
30   single primer-pair capable of amplifying a region of
the E6 gene from HPV types 16, 18, 31 and 33, and
preferably also HPV 45, which thus enables
amplification of all four (preferably five) types in a
single amplification reaction. This could, for
35   example, be achieved with the use of a pair of
degenerate primers or by selection of a region of the
E6 mRNA which is highly conserved across HPV types.

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The E6 primer-pair may correspond to any region
of the E6 mRNA, an may enable amplification of all or
part of the E6 open reading frame and/or the E7 open
reading frame.

The kit may further include primer-pairs suitable
for use in amplification of E6 mRNA from HPV types
other than types 16, 18, 31 and 33, and preferably
also HPV 45. For example, the kit may be supplemented
with E6 primers for detection of an HPV type which is
endemic in a particular geographical area or
population.

The primer-pair(s) suitable for use in
amplification of a region of L1 transcripts should be
capable of amplifying a region of L1 mRNA from at
least the major cancer-associated HPV types 16, 18, 31
and 33, and preferably also HPV 45, and will
preferably be suitable for use in amplification of a
region of L1 mRNAs from substantially all known HPV
types. With the use of such primers it is possible to
test for active transcription of L1 mRNA from multiple
HPV types in a single amplification reaction.

It is possible to design primers capable of
detecting L1 transcripts from multiple HPV types by
selecting regions of the L1 transcript which are
highly conserved.

In a further approach, specificity for multiple
HPV types may be achieved with the use of degenerate
oligonucleotide primers or complex mixtures of
polynucleotides which exhibit minor sequence
variations, preferably corresponding to sites of
sequence variation between HPV genotypes. The
rationale behind the use of such degenerate primers or
mixtures is that the mixture may contain at least one

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primer-pair capable of detecting each HPV type.

In a still further approach specificity for
multiple HPV types may be achieved by incorporating
5   into the primers one or more inosine nucleotides,
preferably at sites of sequence variation between HPV
genotypes.

The E6 and L1 primer-pairs may be supplied in
10   separate containers within the kit, or the L1 primer-
pair(s) may be supplied as a mixture with one or more
E6 primer-pairs in a single container.

The kits may further comprise one or more probes
15   suitable for use in detection of the products of
amplification reactions carried out using the primer-
pairs included within the kit. The probe(s) may be
supplied as a separate reagent within the kit.
Alternatively, the probe(s) may be supplied as a
20   mixture with one or more primer-pairs.

The primers and probes included in the kit are
preferably single stranded DNA molecules. Non-natural
synthetic polynucleotides which retain the ability to
25   base-pair with a complementary nucleic acid molecule
·may also be used, including synthetic oligonucleotides
which incorporate modified bases and synthetic
oligonucleotides wherein the links between individual
nucleosides include bonds other than phosphodiester
30   bonds. The primers and probes may be produced
according to techniques well known in the art, such as
by chemical synthesis using standard apparatus and
protocols for oligonucleotide synthesis.

35   The primers and probes will typically be isolated
single-stranded polynucleotides of no more than 100
bases in length, more typically less than 55 bases in

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length. For the avoidance of doubt it is hereby
stated that the terms "primer" and "probe" exclude
naturally occurring full-length HPV genomes.

5   Several general types of oligonucleotide primers
and probes incorporating HPV-specific sequences may be
included in the kit. Typically, such primers and
probes may comprise additional, non-HPV sequences, for
example sequences which are required for an
10   amplification reaction or which facilitate detection
of the products of the amplification reaction.

The first type of primers are primer 1
oligonucleotides (also referred to herein as NASBA P1
15   primers), which are oligonucleotides of generally
approximately 50 bases in length, containing an
average of about 20 bases at the 3' end that are
complementary to a region of the target mRNA.
Oligonucleotides suitable for use as NASBA P1 primers
20   are denoted "P1/PCR" in Table 1. P1 primer
oligonucleotides have the general structure X_1-SEQ,
wherein SEQ represents an HPV-specific sequence and X_1
is a sequence comprising a promoter that is recognized
by a specific RNA polymerase. Bacteriophage
25   promoters, for example the T7, T3 and SP6 promoters,
. are preferred for use in the oligonucleotides of the
invention, since they provide advantages of high level
transcription which is dependent only on binding of
the appropriate RNA polymerase. In a preferred
30   embodiment, sequence "X_1" may comprise the sequence
AATTCTAATACGACTCACTATAGGG or the sequence
AATTCTAATACGACTCACTATAGGGAGAAGG. These sequences
contains a T7 promoter, including the transcription
initiation site for T7 RNA polymerase.
35   The HPV-specific sequences in the primers denoted in
Table 1 as "P1/PCR" may also be adapted for use in
standard PCR primers. When these sequences are used

as the basis of NASBA P1 primers they have the general
structure X_1-SEQ, as defined above. The promoter
sequence X_1 is essential in a NASBA P1 primer.
However, when the same sequences are used as the basis
5   of standard PCR primers it is not necessary to include
X_1.

A second type of primers are NASBA primer 2
oligonucleotides (also referred to herein as NASBA P2
10 primers) which generally comprise a sequence of
approximately 20 bases substantially identical to a
region of the target mRNA. The oligonucleotide
sequences denoted in Table 1 as "P2/PCR" are suitable
for use in both NASBA P2 primers and standard PCR
15 primers.

Oligonucleotides intended for use as NASBA P2
primers may, in a particular but non-limiting
embodiment, further comprise a sequence of nucleotides
20 at the 5' end which is unrelated to the target mRNA
but which is capable of hybridising to a generic
detection probe. The detection probe will preferably
be labelled, for example with a fluorescent,
luminescent or enzymatic label. In one embodiment the
25 detection probe is labelled with a label that permits
detection using ECL™ technology, although it will be
appreciated that the invention is in no way limited to
this particular method of detection. In a preferred
embodiment the 5' end of the primer 2 oligonucleotides
30 may comprise the sequence GATGCAAGGTCGCATATGAG. This
sequence is capable of hybridising to a generic ECL™
probe commercially available from Organon Teknika
having the following structure:

35 Ru(bpy)$_3^{2+}$-GAT GCA AGG TCG CAT ATG AG-3'

In a different embodiment the primer 2

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oligonucleotide may incorporate "molecular beacons"
technology, which is known in the art and described,
for example, in WO 95/13399 by Tyagi and Kramer,
Nature Biotechnology. 14: 303-308, 1996, to allow for
5   real-time monitoring of the NASBA reaction.

Target-specific probe oligonucleotides may also
be included within the kit. Probe oligonucleotides
generally comprise a sequence of approximately 20-25
10   bases substantially identical to a region of the
target mRNA, or the complement thereof. Example HPV-
specific oligonucleotide sequences which are suitable
for use as probes are denoted "PO" in Table 1. The
probe oligonucleotides may be used as target-specific
15   hybridisation probes for detection of the products of
a NASBA or PCR reaction. In this connection the probe
oligonucleotides may be coupled to a solid support,
such as paramagnetic beads, to form a capture probe
(see below). In a preferred embodiment the 5' end of
20   the probe oligonucleotide may be labelled with biotin.
The addition of a biotin label facilitates attachment
of the probe to a solid support via a
biotin/streptavidin or biotin/avidin linkage.

25   Target-specific probes enabling real-time
.detection of amplification products may incorporate
"molecular beacons" technology which is known in the
art and described, for example, by Tyagi and Kramer,
Nature Biotechnology. 14: 303-308, 1996 and in WO
30   95/13399. Example HPV-specific oligonucleotide
sequences suitable for use as molecular beacons probes
are denoted "MB" in Table 1.

The term "molecular beacons probes" as used
35   herein is taken to mean molecules having the
structure:

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X_2-arm_1-target-arm_2-X_3

wherein "target" represents a target-specific sequence
of nucleotides, "X_2" and "X_3" represent a fluorescent
5   moiety and a quencher moiety capable of substantially
or completely quenching the fluorescence from the
fluorescent moiety when the two are held together in
close proximity and "arm_1" and "arm_2" represent
complementary sequences capable of forming a stem
10   duplex.

Preferred combinations of "arm_1" and "arm_2"
sequences are as follows, however these are intended
to be illustrative rather than limiting to the
15   invention:

cgcatg-SEQ-catgcg
ccagct-SEQ-agctgg
cacgc-SEQ-gcgtg
20   cgatcg-SEQ-cgatcg
ccgtcg-SEQ-cgacgg
cggacc-SEQ-ggtccg
ccgaagg-SEQ-ccttcgg
cacgtcg-SEQ-cgacgtg
25   cgcagc-SEQ-gctgcg
·ccaagc-SEQ-gcttgg
ccaagcg-SEQ-cgcttgg
cccagc-SEQ-gctggg
ccaaagc-SEQ-gctttgg
30   cctgc-SEQ-gcagg
ccaccc-SEQ-gggtgg
ccaagcc-SEQ-ggcttgg
ccagcg-SEQ-cgctgg
cgcatg-SEQ-catgcg

35

The use of molecular beacons technology allows
for real-time monitoring of amplification reactions,

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for example NASBA amplification (see Leone et al.,
Nucleic Acids Research., 1998, vol: 26, pp 2150-2155).
The molecular beacons probes generally include
complementary sequences flanking the HPV-specific
sequence, represented herein by the notation arm_1 and
arm_2, which are capable of hybridising to each other
form a stem duplex structure. The precise sequences
of arm_1 and arm_2 are not material to the invention,
except for the requirement that these sequences must
be capable of forming a stem duplex when the probe is
not bound to a target HPV sequence.

Molecular beacons probes also include a
fluorescent moiety and a quencher moiety, the
fluorescent and the quencher moieties being
represented herein by the notation X_2 and X_3. As will
be appreciated be the skilled reader, the fluorescer
and quencher moieties are selected such that the
quencher moiety is capable of substantially or
completely quenching the fluorescence from the
fluorescent moiety when the two moieties are in close
proximity, e.g. when the probe is in the hairpin
"closed" conformation in the absence of the target
sequence. Upon binding to the target sequence, the
fluorescent and quencher moieties are held apart such
that the fluorescence of the fluorescent moiety is no
longer quenched.

Many examples of suitable pairs of
quencher/fluorescer moieties which may be used in
accordance with the invention are known in the art
(see WO 95/13399, Tyagi and Kramer, ibid). A broad
range of fluorophores in many different colours made
be used, including for example
5-(2'-aminoethyl)aminonaphthalene-1-sulphonic acid
(EDANS), fluorescein, FAM and Texas Red (see Tyagi,
Bratu and Kramer, 1998, Nature Biotechnology, 16, 49-

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53. The use of probes labelled with different
coloured fluorophores enables "multiplex" detection of
two or more different probes in a single reaction
vessel. A preferred quencher is
4-(4'-dimethylaminophenylazo)benzoic acid (DABCYL), a
non-fluorescent chromophore, which serves as a
"universal" quencher for a wide range of fluorophores.
The fluorescer and quencher moieties may be covalently
attached to the probe in either orientation, either
with the fluorescer at or near the 5' end and the
quencher at or near the 3' end or vice versa.
Protocols for the synthesis of molecular beacon probes
are known in the art. A detailed protocol for
synthesis is provided in a paper entitled "Molecular
Beacons: Hybridization Probes for Detection of Nucleic
Acids in Homogenous Solutions" by Sanjay Tyagi et al.,
Department of Molecular Genetics, Public Health
Research Institute, 455 First Avenue, New York, NY
10016, USA, which is available online via the PHRI
website (at www.phri.nyu.edu or www.molecular-
beacons.org)

     Suitable combinations of the NASBA P1 and NASBA
P2 primers may be used to drive a NASBA amplification
reaction. In order to drive a NASBA amplification
reaction the primer 1 and primer 2 oligonucleotides
must be capable of priming synthesis of a
double-stranded DNA from a target region of mRNA. For
this to occur the primer 1 and primer 2
oligonucleotides must comprise target-specific
sequences which are complementary to regions of the
sense and the antisense strand of the target mRNA,
respectively.

     In the first phase of the NASBA amplification
cycle, the so-called "non-cyclic" phase, the primer 1
oligonucleotide anneals to a complementary sequence in

the target mRNA and its 3' end is extended by the
action of an RNA-dependent DNA polymerase (e.g.
reverse transcriptase) to form a first-strand cDNA
synthesis. The RNA strand of the resulting RNA:DNA
5   hybrid is then digested, e.g. by the action of RNaseH,
to leave a single stranded DNA. The primer 2
oligonucleotide anneals to a complementary sequence
towards the 3' end of this single stranded DNA and its
3' end is extended (by the action of reverse
10   transcriptase), forming a double stranded DNA. RNA
polymerase is then able to transcribe multiple RNA
copies from the now transcriptionally active promoter
sequence within the double-stranded DNA. This RNA
transcript, which is antisense to the original target
15   mRNA, can act as a template for a further round of
NASBA reactions, with primer 2 annealing to the RNA
and priming synthesis of the first cDNA strand and
primer 1 priming synthesis of the second cDNA strand.
The general principles of the NASBA reaction are well
20   known in the art (see Compton, J. Nature. 350: 91-92).

The target-specific probe oligonucleotides
described herein may also be attached to a solid
support, such as magnetic microbeads, and used as
25   "capture probes" to immobilise the product of the
NASBA amplification reaction (a single stranded RNA).
The target-specific "molecular beacons" probes
described herein may be used for real-time monitoring
of the NASBA reaction.

30
Kits according to the invention may also
including a positive control containing E6 and/or L1
mRNA from a known HPV type. Suitable controls
include, for example, nucleic acid extracts prepared
35   from cell lines infected with known HPV types (e.g.
HeLa, CaSki).

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Kits further may contain internal control amplification primers, e.g. primers specific for human U1A RNA.

5   Kits containing primers (and optionally probes) suitable for use in NASBA amplification may further comprise a mixture of enzymes required for the NASBA reaction, e.g. enzyme mixture containing an RNA directed DNA polymerase (e.g. a reverse

10   transcriptase), a ribonuclease that hydrolyses the RNA strand of an RNA-DNA hybrid without hydrolysing single or double stranded RNA or DNA (e.g. RNaseH) and an RNA polymerase. The RNA polymerase should be one which recognises the promoter sequence present in the 5'

15   terminal region of the NASBA P1 primers supplied in the reagent kit. The kit may also comprise a supply of NASBA buffer containing the ribonucleosides and deoxyribonucleosides required for RNA and DNA synthesis. The composition of a standard NASBA

20   reaction buffer will be well known to those skilled in the art (see also Leone et al., ibid).

Table 1: E6-specific sequences for inclusion in NASBA/PCR primers and probes

25

SEQ ID	Primer /probe type	Sequence	HPV Type	nt
1	P2/PCR	CCACAGGAGCGACCCAGAAAGTTA	16	116
2	P1/PCR	X_1-ACGGTTTGTTGTATTGCTGTTC	16	368
3	P2/PCR	CCACAGGAGCGACCCAGAAA	16	116
4	P1/PCR	X_1-GGTTTGTTGTATTGCTGTTC	16	368
5	P1/PCR	X_1-ATTCCCATCTCTATATACTA	16	258
6	P1/PCR	X_1-TCACGTCGCAGTAACTGT	16	208
7	P1/PCR	X_1-TTGCTTGCAGTACACACA	16	191
8	P1/PCR	X_1-TGCAGTACACACATTCTA	16	186
9	P1/PCR	X_1-GCAGTACACACATTCTAA	16	185
10	P2/PCR	ACAGTTATGCACAGAGCT	16	142
11	P2/PCR	ATATTAGAATGTGTGTAC	16	182
12	P2/PCR	TTAGAATGTGTGTACTGC	16	185
13	P2/PCR	GAATGTGTGTACTGCAAG	16	188

SEQ ID	Primer /probe type	Sequence	HPV Type	nt
14	PO	ACAGTTATGCACAGAGCT	16	142
15	PO	ATATTAGAATGTGTGTAC	16	182
16	PO	TTAGAATGTGTGTACTGC	16	185
17	PO	GAATGTGTGTACTGCAAG	16	188
18	PO	CTTTGCTTTTCGGGATTTATGC	16	235
19	PO	TATGACTTTGCTTTTCGGGA	16	230
20	MB	X_2-arm_1-TATGACTTTGCTTTTCGGGA-arm_2-X_3	16	230
21	P2/PCR	CAGAGGAGGAGGATGAAATAGTA	16	656
22	P1/PCR	X_1-GCACAACCGAAGCGTAGAGTCACAC	16	741
23	PO	TGGACAAGCAGAACCGGACAGAGC	16	687
24	P2/PCR	CAGAGGAGGAGGATGAAATAGA	16	656
25	P1/PCR	X_1-GCACAACCGAAGCGTAGAGTCA	16	741
26	PO	AGCAGAACCGGACAGAGCCCATTA	16	693
27	P2/PCR	ACGATGAAATAGATGGAGTT	18	702
28	P1/PCR	X_1-CACGGACACACAAAGGACAG	18	869
28	PO	AGCCGAACCACAACGTCACA	18	748
30	P2/PCR	GAAAACGATGAAATAGATGGAG	18	698
31	P1/PCR	X_1-ACACCACGGACACACAAAGGACAG	18	869
32	PO	GAACCACAACGTCACACAATG	18	752
33	MB	X_2-arm_1-GAACCACAACGTCACACAATG-arm_2-X_3	18	752
34	P2/PCR	TTCCGGTTGACCTTCTATGT	18	651
35	P1/PCR	X_1-GGTCGTCTGCTGAGCTTTCT	18	817
36	P2/PCR	GCAAGACATAGAAATAACCTG	18	179
37	P1/PCR	X_1-ACCCAGTGTTAGTTAGTT	18	379
38	PO	TGCAAGACAGTATTGGAACT	18	207
39	P2/PCR	GGAAATACCCTACGATGAAC	31	164
40	P1/PCR	X_1-GGACACAACGGTCTTTGACA	31	423
41	PO	ATAGGGACGACACACCACGGAG	31	268
42	P2/PCR	GGAAATACCCTACGATGAACTA	31	164
43	P1/PCR	X_1-CTGGACACAACGGTCTTTGACA	31	423
44	PO	TAGGGACGACACACCACACGGA	31	269
45	P2/PCR	ACTGACCTCCACTGTTATGA	31	617
46	P1/PCR	X_1-TATCTACTTGTGTGCTCTGT	31	766
47	PO	GACAAGCAGAACCGGACACATC	31	687
48	P2/PCR	TGACCTCCACTGTTATGAGCAATT	31	619
49	P1/PCR	X_1-TGCGAATATCTACTTGTGTGCTCT GT	31	766
50	PO	GGACAAGCAGAACCGGACACATCCAA	31	686
51	MB	X_2-arm_1-GGACAAGCAGAACCGGACACATCCAA-arm_2-X_3	31	686
52	P2/PCR	ACTGACCTCCACTGTTAT	31	617
53	P1/PCR	X_1-CACGATTCCAAATGAGCCCAT	31	809
54	P2/PCR	TATCCTGAACCAACTGACCTAT	33	618
55	P1/PCR	X_1-TTGACACATAAACGAACTG	33	763
56	PO	CAGATGGACAAGCACAACC	33	694
57	P2/PCR	TCCTGAACCAACTGACCTAT	33	620
58	P1/PCR	X_1-CCCATAAGTAGTTGCTGTAT	33	807
59	PO	GGACAAGCACAACCAGCCACAGC	33	699
60	MB	X_2-arm_1-GGACAAGCACAACCAGCCACAGC-arm_2-X_3	33	699

SEQ ID	Primer /probe type	Sequence	HPV Type	nt
61	P2/PCR	GACCTTTGTGTCCTCAAGAA	33	431
62	P1/PCR	X_1-AGGTCAGTTGGTTCAGGATA	33	618
63	PO	AGAAACTGCACTGTGACGTGT	33	543
64	P2/PCR	ATTACAGCGGAGTGAGGTAT	35	217
65	P1/PCR	X_1-GTCTTTGCTTTTCAACTGGA	35	442
66	PO	ATAGAGAAGGCCAGCCATAT	35	270
67	P2/PCR	TCAGAGGAGGAGGAAGATACTA	35	655
68	P1/PCR	X_1-GATTATGCTCTCTGTGAACA	35	844
69	P2/PCR	CCCGAGGCAACTGACCTATA	35	610
70	P1/PCR	X_1-GTCAATGTGTGTGCTCTGTA	35	770
71	PO	GACAAGCAAAACCAGACACCTCCAA	35	692
72	PO	GACAAGCAAAACCAGACACC	35	692
73	P2/PCR	TTGTGTGAGGTGCTGGAAGAAT	52	144
74	P1/PCR	X_1-CCCTCTCTTCTAATGTTT	52	358
75	PO	GTGCCTACGCTTTTTATCTA	52	296
76	P2/PCR	GTGCCTACGCTTTTTATCTA	52	296
77	P1/PCR	X_1-GGGGTCTCCAACACTCTGAACA	52	507
78	PO	TGCAAACAAGCGATTTCA	52	461
79	P2/PCR	TCAGGCGTTGGAGACATC	58	157
80	P1/PCR	X_1-AGCAATCGTAAGCACACT	58	301
81	P2/PCR	TCTGTGCATGAAATCGAA	58	173
82	P1/PCR	X_1-AGCACACTTTACATACTG	58	291
83	PO	TGAAATGCGTTGAATGCA	58	192
84	PO	TTGCAGCGATCTGAGGTATATG	58	218
85	P2/PCR	TACACTGCTGGACAACAT	B(11)	514
86	P1/PCR	X_1-TCATCTTCTGAGCTGTCT	B(11)	619
87	P2/PCR	TACACTGCTGGACAACATGCA	B(11)	514
88	P1/PCR	X_1-GTCACATCCACAGCAACAGGTCA	B(11)	693
89	PO	GTAGGGTTACATTGCTATGA	B(11)	590
90	PO	GTAGGGTTACATTGCTATGAGC	B(11)	590
91	P2/PCR	TGACCTGTTGCTGTGGATGTGA	B(11)	693
92	P1/PCR	X_1-TACCTGAATCGTCCGCCAT	B(11)	832
93	PO	ATWGTGTGTCCCATCTGC	B(11)	794
94	P2/PCR	CATGCCATAAATGTATAGA	C(18 39 45)	295
95	P1/PCR	X_1-CACCGCAGGCACCTTATTAA	C(18 39 45	408
96	PO	AGAATTAGAGAATTAAGA	C(18 39 45	324
97	P2/PCR	GCAGACGACCACTACAGCAAA	39	210
98	P1/PCR	X_1-ACACCGAGTCCGAGTAATA	39	344
99	PO	ATAGGGACGGGGAACCACT	39	273
100	P2/PCR	TATTACTCGGACTCGGTGT	39	344
101	P1/PCR	X_1-CTTGGGTTTCTCTTCGTGTTA	39	558
102	PO	GGACCACAAAACGGGAGGAC	39	531
103	P2/PCR	GAAATAGATGAACCCGACCA	39	703
104	P1/PCR	X_1-GCACACCACGGACACACAAA	39	886
105	PO	TAGCCAGACGGGATGAACCACAGC	39	749
106	P2/PCR	AACCATTGAACCCAGCAGAAA	45	430

SEQ ID	Primer /probe type	Sequence	HPV Type	nt
107	P1/PCR	X₁-TCTTTCTTGCCGTGCCTGGTCA	45	527
108	PO	GTACCGAGGGCAGTGTAATA	45	500
109	P2/PCR	AACCATTGAACCCAGCAGAAA	45	430
110	P1/PCR	X₁-TCTTTCTTGCCGTGCCTGGTCA	45	527
111	P2/PCR	GAAACCATTGAACCCAGCAGAAAA	45	428
112	P1/PCR	X₁-TTGCTATACTTGTGTTTCCCTACG	45	558
113	PO	GTACCGAGGGCAGTGTAATA	45	500
114	PO	GGACAAACGAAGATTTCACA	45	467
115	P2/PCR	GTTGACCTGTTGTGTTACCAGCAAT	45	656
116	P1/PCR	X₁-CACCCACGGACACACAAAGGACAAG	45	868
117	P2/PCR	CTGTTGACCTGTTGTGTTACGA	45	654
118	P1/PCR	X₁-CCACGGACACACAAAGGACAAG	45	868
119	P2/PCR	GTTGACCTGTTGTGTTACGA	45	656
120	P1/PCR	X₁-ACGGACACACAAAGGACAAG	45	868
121	PO	GAGTCAGAGGAGGAAAACGATG	45	686
122	PO	AGGAAAACGATGAAGCAGATGGAGT	45	696
123	PO	ACAACTACCAGCCCGACGAGCCGAA	45	730
124	P2/PCR	GGAGGAGGATGAAGTAGATA	51	658
125	P1/PCR	X₁-GCCCATTAACATCTGCTGTA	51	807
126	P2/PCR	AGAGGAGGAGGATGAAGTAGATA	51	655
127	P1/PCR	X₁-ACGGGCAAACCAGGCTTAGT	51	829
128	PO	GCAGGTGTTCAAGTGTAGTA	51	747
129	PO	TGGCAGTGGAAAGCAGTGGAGACA	51	771
130	P2/PCR	TTGGGGTGCTGGAGACAAACATCT	56	519
131	P1/PCR	X₁-TTCATCCTCATCCTCATCCTCTGA	56	665
132	P2/PCR	TGGGGTGCTGGAGACAAACATC	56	520
133	P1/PCR	X₁-CATCCTCATCCTCATCCTCTGA	56	665
134	P2/PCR	TTGGGGTGCTGGAGACAAACAT	56	519
135	P1/PCR	X₁-CCACAAACTTACACTCACAACA	56	764
136	PO	AAAGTACCAACGCTGCAAGACGT	56	581
137	PO	AGAACTAACACCTCAAACAGAAAT	56	610
138	PO	AGTACCAACGCTGCAAGACGTT	56	583
139	P1/PCR	X₁-TTGGACAGCTCAGAGGATGAGG	56	656
140	P2/PCR	GATTTTCCTTATGCAGTGTG	56	279
141	P1/PCR	X₁-GACATCTGTAGCACCTTATT	56	410
142	PO	GACTATTCAGTGTATGGAGC	56	348
143	PO	CAACTGAYCTMYACTGTTATGA	A (16 31 35)	
144	MB	X₂-arm₁-CAACTGAYCTMYACTGTTATGA-arm₂-X₃	A (16 31 35)	
145	PO	GAAMCAACTGACCTAYWCTGCTAT	A (33 52 58)	
146	MB	X₂-arm₁-GAAMCAACTGACCTAYWCTGCTAT-arm₂-X₃	A (33 52 58)	
147	PO	AAGACATTATTCAGACTC	C (18 45 39)	
148	MB	X₂-arm₁-AAGACATTATTCAGACTC-arm₂-X₃	C (18 45 39)	

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Table 2: L1-specific sequences for inclusion in
NASBA/PCR primers and probes

SEQ ID	Primer/probe type	Sequence
149	P2/PCR	AATGGCATTTGTTGGGGTAA
150	P1/PCR	X₁-TCATATTCCTCCCCCATGTC
151	PO	TTGTTACTGTTGTTGATACTAC
152	P2/PCR	AATGGCATTTGTTGGSRHAA
153	P1/PCR	X₁-TCATATTCCTCMMCATGDC
154	PO	TTGTTACTGTTGTTGATACYAC
155	PO	TTGTTACTGTTGTTGATACCAC
156	P2/PCR	AATGGCATTTGTTGGSIIAA
157	P2/PCR	AATGGCATTTGTTGGIIHAA
158	P2/PCR	AATGGCATTTGTTGGIRIAA
159	P2/PCR	AATGGCATTTGTTGGGGTAA
160	P2/PCR	AATGGCATTTGTTGGGGAAA
161	P2/PCR	AATGGCATTTGTTGGCATAA
162	P2/PCR	AATGGCATTTGTTGGGGCAA
163	P2/PCR	AATGGCATTTGTTGGCACAA
164	P1/PCR	X₁-TCATATTCCTCMICATGIC
165	P1/PCR	X₁-TCATATTCCTCAACATGIC
166	P1/PCR	X₁-TCATATTCCTCIICATGTC
167	P1/PCR	X₁-TCATATTCCTCIICATGGC
168	P1/PCR	X₁-TCATATTCCTCIICATGAC 3'
169	P1/PCR	X₁-TCATATTCCTCIICATGCC 3'

Preferred primers suitable for use in detection
of HPV L1 and E6 mRNA by NASBA are listed in the
following tables. However, these are merely
illustrative and it is not intended that the scope of

the invention should be limited to these specific
molecules.

In the following Tables the NASBA P2 primers (p2)
include the sequence GATGCAAGGTCGCATATGAG at the 5'
end; the NASBA P1 primers (p1) include the sequence
AATTCTAATACGACTCACTATAGGGAGAAGG at the 5' end.
Oligonucleotides suitable for use as probes are
identified by "po". The P2 primers generally contain
HPV sequences from the postive strand, whereas the p1
primers generally contain HPV sequences from the
negative strand. nt-refers to nucleotide position in
the relevant HPV genomic sequence.

Table 3-Preferred E6 NASBA primers and probes

Primer name	Sequence	HPV Type	nt
HAe6701p2	GATGCAAGGTCGCATATGAGCCACAGGAGCGACCCAG AAAGTTA	16	116
HAe6701p1	AATTCTAATACGACTCACTATAGGGAGAAGGACGGTT TGTTGTATTGCTGTTC	16	368
HAe6702p2	GATGCAAGGTCGCATATGAGCCACAGGAGCGACCCAG AAA	16	116
HAe6702p1	AATTCTAATACGACTCACTATAGGGAGAAGGGGTTTG TTGTATTGCTGTTC	16	368
HPV16p1	AATTCTAATACGACTCACTATAGGGAGAAGGATTCCC ATCTCTATATACTA	16	258
HAe6702Ap1	AATTCTAATACGACTCACTATAGGGAGAAGGTCA CGTCGCAGTAACTGT	16	208
HAe6702Bp1	AATTCTAATACGACTCACTATAGGGAGAAGGTTG CTTGCAGTACACACA	16	191
HAe6702Cp1	AATTCTAATACGACTCACTATAGGGAGAAGGTGC AGTACACACATTCTA	16	186
HAe6702Dp1	AATTCTAATACGACTCACTATAGGGAGAAGGGCA GTACACACATTCTAA	16	185
H16e6702Ap2	GATGCAAGGTCGCATATGAGACAGTTATGCACAGAGCT	16	142
H16e6702Bp2	GATGCAAGGTCGCATATGAGATATTAGAATGTGTGTAC	16	182
H16e6702Cp2	GATGCAAGGTCGCATATGAGTTAGAATGTGTGTACTGC	16	185
H16e6702Dp2	GATGCAAGGTCGCATATGAGGAATGTGTGTACTGCAAG	16	188
H16e6702Apo	ACAGTTATGCACAGAGCT	16	142
H16e6702Bpo	ATATTAGAATGTGTGTAC	16	182
H16e6702Cpo	TTAGAATGTGTGTACTGC	16	185

Primer name	Sequence	HPV Type	nt
H16e6702Dpo	GAATGTGTGTACTGCAAG	16	188
HAe6701po	CTTTGCTTTTCGGGATTTATGC	16	235
HAe6702po	TATGACTTTGCTTTTCGGGA	16	230
HAe6702mb1	X₂-cgcatgTATGACTTTGCTTTTCGGGAcatgcg -X₃	16	230
HAe6702mb2	X₂-ccagctTATGACTTTGCTTTTCGGGAagctgg -X₃	16	230
HAe6702mb3	X₂-cacgcTATGACTTTGCTTTTCGGGAgcgtg -X₃	16	230
H16e6702mb4	X₂-cgatcgTATGACTTTGCTTTTCGGGAcgatcg -X₃	16	230
HAe6703p2	GATGCAAGGTCGCATATGAGCAGAGGAGGAGGATGAA ATAGTA	16	656
HAe6703p1	AATTCTAATACGACTCACTATAGGGAGAAGGGCACAA CCGAAGCGTAGAGTCACAC	16	741
HAe6703po	TGGACAAGCAGAACCGGACAGAGC	16	687
HAe6704p2	GATGCAAGGTCGCATATGAGCAGAGGAGGAGGATGAA ATAGA	16	656
HAe6704p1	AATTCTAATACGACTCACTATAGGGAGAAGGGCACAA CCGAAGCGTAGAGTCA	16	741
HAe6704po	AGCAGAACCGGACAGAGCCCATTA	16	693
H18e6701p2	GATGCAAGGTCGCATATGAGACGATGAAATAGATGGA GTT	18	702
H18e6701p1	AATTCTAATACGACTCACTATAGGGAGAAGGCACGGA CACACAAAGGACAG	18	869
H18e6701po	AGCCGAACCACAACGTCACA	18	748
H18e6702p2	GATGCAAGGTCGCATATGAGGAAAACGATGAAATAGA TGGAG	18	698
H18e6702p1	AATTCTAATACGACTCACTATAGGGAGAAGGACACCA CGGACACACAAAGGACAG	18	869
H18e6702po	GAACCACAACGTCACACAATG	18	752
H18e6702mb1	X₂-cgcatgGAACCACAACGTCACACAATGcatgcg -X₃	18	752
H18e6702mb2	X₂-ccgtcgGAACCACAACGTCACACAATGcgacgg -X₃	18	752
H18e6702mb3	X₂-cggaccGAACCACAACGTCACACAATGggtccg -X₃	18	752
H18e6702mb4	X₂-cgatcgGAACCACAACGTCACACAATGcgatcg -X₃	18	752
H18e6703p2	GATGCAAGGTCGCATATGAGTTCCGGTTGACCTTCTA TGT	18	651
H18e6703p1	AATTCTAATACGACTCACTATAGGGAGAAGGGGTCGT CTGCTGAGCTTTCT	18	817
H18e6704p2	GATGCAAGGTCGCATATGAGGCAAGACATAGAAATAA CCTG	18	179
H18e6704p1	AATTCTAATACGACTCACTATAGGGAGAAGGACCCAG TGTTAGTTAGTT	18	379
H18e6704po	TGCAAGACAGTATTGGAACT	18	207
H31e6701p2	GATGCAAGGTCGCATATGAGGGAAATACCCTACGATG AAC	31	164

Primer name	Sequence	HPV Type	nt
H31e6701p1	AATTCTAATACGACTCACTATAGGGAGAAGGGGACAC AACGGTCTTTGACA	31	423
H31e6701po	ATAGGGACGACACACCACACGGAG	31	268
H31e6702p2	GATGCAAGGTCGCATATGAGGGAAATACCCTACGATG AACTA	31	164
H31e6702p1	AATTCTAATACGACTCACTATAGGGAGAAGGCTGGAC ACAACGGTCTTTGACA	31	423
H31e6702po	TAGGGACGACACACCACACGGA	31	269
H31e6703p2	GATGCAAGGTCGCATATGAGACTGACCTCCACTGTTA TGA	31	617
H31e6703p1	AATTCTAATACGACTCACTATAGGGAGAAGGTATCTA CTTGTGTGCTCTGT	31	766
H31e6703po	GACAAGCAGAACCGGACACATC	31	687
H31e6704p2	GATGCAAGGTCGCATATGAGTGACCTCCACTGTTATG AGCAATT	31	619
H31e6704p1	AATTCTAATACGACTCACTATAGGGAGAAGGTGCGAA TATCTACTTGTGTGCTCT GT	31	766
H31e6704po	GGACAAGCAGAACCGGACACATCCAA	31	686
H31e6704mb1	X₂-ccgaaggGGACAAGCAGAACCGGACACATCC AAccttcgg -X₃	31	686
H31e6704mb2	X₂-ccgtcgGGACAAGCAGAACCGGACACATCCA Acgacgg -X₃	31	686
H31e6704mb3	X₂-cacgtcgGGACAAGCAGAACCGGACACATCCAA cgacgtg -X₃	31	686
H31e6704mb4	X₂-cgcagcGGACAAGCAGAACCGGACACATCCAA gctgcg -X₃	31	686
H31e6704mb5	X₂-cgatcgGGACAAGCAGAACCGGACACATCCAA cgatcg -X₃	31	686
H31e6705p2	GATGCAAGGTCGCATATGAGACTGACCTCCACTGTTAT	31	617
H31e6705p1	AATTCTAATACGACTCACTATAGGGAGAAGGCACGAT TCCAAATGAGCCCAT	31	809
H33e6701p2	GATGCAAGGTCGCATATGAGTATCCTGAACCAACTGA CCTAT	33	618
H33e6701p1	AATTCTAATACGACTCACTATAGGGAGAAGGTTGACA CATAAACGAACTG	33	763
H33e6701po	CAGATGGACAAGCACAACC	33	694
H33e6703p2	GATGCAAGGTCGCATATGAGTCCTGAACCAACTGACC TAT	33	620
H33e6703p1	AATTCTAATACGACTCACTATAGGGAGAAGGCCCATA AGTAGTTGCTGTAT	33	807
H33e6703po	GGACAAGCACAACCAGCCACAGC	33	699
H33e6703mb1	X₂-ccaagcGGACAAGCACAACCAGCCACAGCgct tgg -X₃	33	699
H33e6703mb2	X₂-ccaagcgGGACAAGCACAACCAGCCACAGC cgcttgg -X₃	33	699
H33e6703mb3	X₂-cccagcGGACAAGCACAACCAGCCACAGCgct	33	699

Primer name	Sequence	HPV Type	nt
	ggg -X₃		
H33e6703mb4	X₂-ccaaagcGGACAAGCACAACCAGCCACAGCg ctttgg -X₃	33	699
H33e6703mb5	X₂-cctgcGGACAAGCACAACCAGCCACAGCgcagg -X₃	33	699
H33e6703mb6	X₂-cgatcgGGACAAGCACAACCAGCCACAGCga tcg -X₃	33	699
H33e6702p2	GATGCAAGGTCGCATATGAGGACCTTTGTGTCCTCAA GAA	33	431
H33e6702p1	AATTCTAATACGACTCACTATAGGGAGAAGGAGGTCA GTTGGTTCAGGATA	33	618
H33e6702po	AGAAACTGCACTGTGACGTGT	33	543
H35e6701p2	GATGCAAGGTCGCATATGAGATTACAGCGGAGTGAGG TAT	35	217
H35e6701p1	AATTCTAATACGACTCACTATAGGGAGAAGGGTCTTT GCTTTTCAACTGGA	35	442
H35e5601po	ATAGAGAAGGCCAGCCATAT	35	270
H35e6702p2	GATGCAAGGTCGCATATGAGTCAGAGGAGGAGGAAGA TACTA	35	655
H35e6702p1	AATTCTAATACGACTCACTATAGGGAGAAGGGATTAT GCTCTCTGTGAACA	35	844
H35e6703p2	GATGCAAGGTCGCATATGAGCCCGAGGCAACTGACCT ATA	35	610
H35e6703p1	AATTCTAATACGACTCACTATAGGGAGAAGGGTCAAT GTGTGTGCTCTGTA	35	770
H35e6702po	GACAAGCAAAACCAGACACCTCCAA	35	692
H35e6703po	GACAAGCAAAACCAGACACC	35	692
H52e6701p2	GATGCAAGGTCGCATATGAGTTGTGTGAGGTGCTGGA AGAAT	52	144
H52e6701p1	AATTCTAATACGACTCACTATAGGGAGAAGGCCCTCT CTTCTAATGTTT	52	358
H52e6701po	GTGCCTACGCTTTTTATCTA	52	296
H52e6702p2	GATGCAAGGTCGCATATGAGGTGCCTACGCTTTTTAT CTA	52	296
H52e6702p1	AATTCTAATACGACTCACTATAGGGAGAAGGGGGGTC TCCAACACTCTGAACA	52	507
H52e6702po	TGCAAACAAGCGATTTCA	52	461
H58e6701p2	GATGCAAGGTCGCATATGAGTCAGGCGTTGGAGACATC	58	157
H58e6701p1	AATTCTAATACGACTCACTATAGGGAGAAGGAGCAAT CGTAAGCACACT	58	301
H58e6702p2	GATGCAAGGTCGCATATGAGTCTGTGCATGAAATCGAA	58	173
H58e6702p1	AATTCTAATACGACTCACTATAGGGAGAAGGAGCACA CTTTACATACTG	58	291
H58e6701po	TGAAATGCGTTGAATGCA	58	192
H58e6702po	TTGCAGCGATCTGAGGTATATG	58	218
HBe6701p2	GATGCAAGGTCGCATATGAGTACACTGCTGGACAACAT	B(11)	514
HBe6701p1	AATTCTAATACGACTCACTATAGGGAGAAGGTCATCT	B(11)	619

Primer name	Sequence	HPV Type	nt
	TCTGAGCTGTCT		
HBe6702p2	GATGCAAGGTCGCATATGAGTACACTGCTGGACAACA TGCA	B(11)	514
HBe6702p1	AATTCTAATACGACTCACTATAGGGAGAAGGGTCACA TCCACAGCAACAGGTCA	B(11)	693
HBe6701po	GTAGGGTTACATTGCTATGA	B(11)	590
HBe6702po	GTAGGGTTACATTGCTATGAGC	B(11)	590
HBe6703p2	GATGCAAGGTCGCATATGAGTGACCTGTTGCTGTGGA TGTGA	B(11)	693
HBe6703p1	AATTCTAATACGACTCACTATAGGGAGAAGGTACCTG AATCGTCCGCCAT	B(11)	832
HBe6703po	ATWGTGTGTCCCATCTGC	B(11)	794
HCe6701p2	GATGCAAGGTCGCATATGAGCATGCCATAAATGTATAGA	C(18 39 45)	295
HCe6701p1	AATTCTAATACGACTCACTATAGGGAGAAGGCACCGC AGGCACCTTATTAA	C(18 39 45	408
HCe6701po	AGAATTAGAGAATTAAGA	C(18 39 45	324
H39e6701p2	GATGCAAGGTCGCATATGAGGCAGACGACCACTACAG CAAA	39	210
H39e6701p1	AATTCTAATACGACTCACTATAGGGAGAAGGACACCG AGTCCGAGTAATA	39	344
H39e6701po	ATAGGGACGGGGAACCACT	39	273
H39e6702p2	GATGCAAGGTCGCATATGAGTATTACTCGGACTCGGTGT	39	344
H39e6702p1	AATTCTAATACGACTCACTATAGGGAGAAGGCTTGGG TTTCTCTTCGTGTTA	39	558
H39e6702po	GGACCACAAAACGGGAGGAC	39	531
H39e6703p2	GATGCAAGGTCGCATATGAGGAAATAGATGAACCCGA CCA	39	703
H39e6703p1	AATTCTAATACGACTCACTATAGGGAGAAGGGCACAC CACGGACACACAAA	39	886
H39e6703po	TAGCCAGACGGGATGAACCACAGC	39	749
HPV45p2	GATGCAAGGTCGCATATGAGAACCATTGAACCCAGCA GAAA	45	430
HPV45p1	AATTCTAATACGACTCACTATAGGGAGAAGGTCTTTC TTGCCGTGCCTGGTCA	45	527
HPV45po	GTACCGAGGGCAGTGTAATA	45	500
H45e6701p2	GATGCAAGGTCGCATATGAGAACCATTGAACCCAGCA GAAA	45	430
H45e6701p1	AATTCTAATACGACTCACTATAGGGAGAAGGTCTTTC TTGCCGTGCCTGGTCA	45	527
H45e6702p2	GATGCAAGGTCGCATATGAGGAAACCATTGAACCCAG CAGAAAA	45	428
H45e6702p1	AATTCTAATACGACTCACTATAGGGAGAAGGTTGCTA TACTTGTGTTTCCCTACG	45	558
H45e6701po	GTACCGAGGGCAGTGTAATA	45	500
H45e6702po	GGACAAACGAAGATTTCACA	45	467

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Primer name	Sequence	HPV Type	nt
H45e6703p2	GATGCAAGGTCGCATATGAGGTTGACCTGTTGTGTTA CCAGCAAT	45	656
H45e6703p1	AATTCTAATACGACTCACTATAGGGAGAAGGCACCAC GGACACACAAAGGACAAG	45	868
H45e6704p2	GATGCAAGGTCGCATATGAGCTGTTGACCTGTTGTGT TACGA	45	654
H45e6704p1	AATTCTAATACGACTCACTATAGGGAGAAGGCCACGG ACACACAAAGGACAAG	45	868
H45e6705p2	GATGCAAGGTCGCATATGAGGTTGACCTGTTGTGTTA CGA	45˙	656
H45e6705p1	AATTCTAATACGACTCACTATAGGGAGAAGGACGGAC ACACAAAGGACAAG	45	868
H45e6703po	GAGTCAGAGGAGGAAAACGATG	45	686
H45e6704po	AGGAAAACGATGAAGCAGATGGAGT	45	696
H45e6705po	ACAACTACCAGCCCGACGAGCCGAA	45	730
H51e6701p2	GATGCAAGGTCGCATATGAGGGAGGAGGATGAAGTAG ATA	51	658
H51e6701p1	AATTCTAATACGACTCACTATAGGGAGAAGGGCCCAT TAACATCTGCTGTA	51	807
H51e6702p2	GATGCAAGGTCGCATATGAGAGAGGAGGAGGATGAAG TAGATA	51	655
H51e6702p1	AATTCTAATACGACTCACTATAGGGAGAAGGACGGGC AAACCAGGCTTAGT	51	829
H51e6701po	GCAGGTGTTCAAGTGTAGTA	51	747
H51e6702po	TGGCAGTGGAAAGCAGTGGAGACA	51	771
H56e6701p2	GATGCAAGGTCGCATATGAGTTGGGGTGCTGGAGACA AACATCT	56	519
H56e6701p1	AATTCTAATACGACTCACTATAGGGAGAAGGTTCATC CTCATCCTCATCCTCTGA	56	665
H56e6702p2	GATGCAAGGTCGCATATGAGTGGGGTGCTGGAGACAA ACATC	56	520
H56e6702p1	AATTCTAATACGACTCACTATAGGGAGAAGGCATCCT CATCCTCATCCTCTGA	56	665
H56e6703p2	GATGCAAGGTCGCATATGAGTTGGGGTGCTGGAGACA AACAT	56	519
H56e6703p1	AATTCTAATACGACTCACTATAGGGAGAAGGCCACAA ACTTACACTCACAACA	56	764
H56e6701po	AAAGTACCAACGCTGCAAGACGT	56	581
H56e6702po	AGAACTAACACCTCAAACAGAAAT	56	610
H56e6703po	AGTACCAACGCTGCAAGACGTT	56	583
H56e6703po1	TTGGACAGCTCAGAGGATGAGG	56	656
H56e6704p2	GATGCAAGGTCGCATATGAGGATTTTCCTTATGCAGT GTG	56	279
H56e6704p1	AATTCTAATACGACTCACTATAGGGAGAAGGGACATC TGTAGCACCTTATT	56	410
H56e6704po	GACTATTCAGTGTATGGAGC	56	348
HPVAPO1A	CAACTGAYCTMYACTGTTATGA	A (16	

Primer name	Sequence	HPV Type	nt
		31 35)	
HPVApo1Amb1	X₂-cgcatgCAACTGAYCTMYACTGTTATGAcatgcg -X₃	A (16 31 35)	
HPVApo1Amb2	X₂-ccgtcgCAACTGAYCTMYACTGTTATGAcga cgg -X₃	A (16 31 35)	
HPVApo1Amb3	X₂-ccacccCAACTGAYCTMYACTGTTATGAgg gtgg -X₃	A (16 31 35)	
HPVApo1Amb4	X₂-cgatcgCAACTGAYCTMYACTGTTATGAcga tcg -X₃	A (16 31 35)	
HPVAPO4A	GAAMCAACTGACCTAYWCTGCTAT	A (33 52 58)	
HPVAPO4Amb1	X₂-ccaagcGAAMCAACTGACCTAYWCTGCTATgc ttgg -X₃	A (33 52 58)	
HPVAPO4Amb2	X₂-ccaagccGAAMCAACTGACCTAYWCTGCTAT ggcttgg -X₃	A (33 52 58)	
HPVAPO4Amb3	X₂-ccaagcgGAAMCAACTGACCTAYWCTGCTA Tcgcttgg -X₃	A (33 52 58)	
HPVAPO4Amb4	X₂-ccagcgGAAMCAACTGACCTAYWCTGCTATcg ctgg -X₃	A (33 52 58)	
HPVAPO4Amb5	X₂-cgatcgGAAMCAACTGACCTAYWCTGCTATcg atcg -X₃	A (33 52 58)	
HPVCPO4	AAGACATTATTCAGACTC	C (18 45 39)	
HPVCPO4Amb1	X₂-ccaagcAAGACATTATTCAGACTCgcttgg -X₃	C (18 45 39)	
HPVCPO4Amb2	X₂-cgcatgAAGACATTATTCAGACTCcatgcg -X₃	C (18 45 39)	
HPVCPO4Amb3	X₂-cccagcAAGACATTATTCAGACTCgctggg -X₃	C (18 45 39)	
HPVCPO4Amb4	X₂-cgatcgAAGACATTATTCAGACTCcgatcg -X₃	C (18 45 39)	

Pairs of P1 and P2 primers having the same prefix
(e.g. HAe6701p1 and HAe6701p2) are intended to be used
in combination. However, other combinations may also
be used, as summarised below for HPV types 16, 18, 31,
33 and 45.

Suitable primer-pairs for amplification of HPV 16 E6
mRNA are as follows:

- 52 -

HAe6701p2 or HAe6702p2 (both nt 116) with HAe6701p1 or
HAe6702p1 (both nt 368).

HAe6701p2 or HAe6702p2 (both nt 116) with HPV16p1 (nt
5   258).

H16e6702Ap2 (nt 142), H16e6702Bp2 (nt 182),
H16e6702Cp2 (nt 185) or H16e6702Dp2 (nt 188) with
HAe6701p1 or HAe6702p1 (both nt 368).
10

HAe6701p2 or HAe6702p2 (both nt 116) with HAe6702Ap1
(nt 208), HAe6702Bp1 (nt 191), HAe6702Cp1 (nt 186) or
HAe6702Dp1 (185). These combinations are suitable for
amplification of all E6 splice variants.
15

HAe6703p2 or HAe6704p2 (both nt 656) with HAe6703p1 or
HAe6704p1 (both nt 741). These combinations are
suitable for amplification of all transcripts
containing the E7 coding region (at least up to nt
20   741).

The following primer-pairs are preferred for
amplification of HPV 18 E6 mRNA:

25   H18e6701p2 (nt 702) or H18e6702p2 (nt 698) with
H18e6701p1 or H18e6702p1 (both nt 869).

H18e6703p2 (nt 651) with H18e6703p1 (nt 817).

30   H18e6704p2 (nt 179) with H18e6704p1 (nt 379).

The following primer-pairs are preferred for
amplification of HPV 31 E6 mRNA:

- 53 -

H31e6701p2 or H31e6702p2 (both nt 164) with H31e6701p1
or H31e6702p1 (both nt 423).

H31e6703p2 (nt 617), H31e6704p2 (nt 619) or H31e6705p2
5   (nt 617) with H31e6703p1 (nt 766), H31e6704p1 (766) or
H31e6705p1 (nt 809).

The following primer-pairs are preferred for
amplification of HPV 33 E6 mRNA:

10

H33e6701p2 (nt 618) or H33e6703p2 (nt 620) with
H33e6701p1 (nt 763) or H33e6703p1 (nt 807).

H33e6702p2 (nt 431) with H33e6702p1 (nt 618).

15

The following primer pair is preferred for
amplification of HPV 45:

HPV45p2 (nt 430) with HPV45p1 (nt 527)

20

Table 4-E6 PCR primers

Primer name	Sequence	HPV type	nt
HAe6701PCR2	CCACAGGAGCGACCCAGAAAGTTA	16	116
HAe6701PCR1	ACGGTTTGTTGTATTGCTGTTC	16	368
HAe6702PCR2	CCACAGGAGCGACCCAGAAA	16	116
HAe6702PCR1	GGTTTGTTGTATTGCTGTTC	16	368
HAe6703PCR2	CAGAGGAGGAGGATGAAATAGTA	16	656
HAe6703PCR1	GCACAACCGAAGCGTAGAGTCACAC	16	741
HAe6704PCR2	CAGAGGAGGAGGATGAAATAGA	16	656
HAe6704PCR1	GCACAACCGAAGCGTAGAGTCA	16	741
H18e6701PCR2	ACGATGAAATAGATGGAGTT	18	702
H18e6701PCR1	CACGGACACACAAAGGACAG	18	869
H18e6702PCR2	GAAAACGATGAAATAGATGGAG	18	698
H18e6702PCR1	ACACCACGGACACACAAAGGACAG	18	869
H18e6703PCR2	TTCCGGTTGACCTTCTATGT	18	651
H18e6703PCR1	GGTCGTCTGCTGAGCTTTCT	18	817
H18e6704PCR2	GCAAGACATAGAAATAACCTG	18	179

Primer name	Sequence	HPV type	nt
H18e6704PCR1	ACCCAGTGTTAGTTAGTT	18	379
H31e6701PCR2	GGAAATACCCTACGATGAAC	31	164
H31e6701PCR1	GGACACAACGGTCTTTGACA	31	423
H31e6702PCR2	GGAAATACCCTACGATGAACTA	31	164
H31e6702PCR1	CTGGACACAACGGTCTTTGACA	31	423
H31e6703PCR2	ACTGACCTCCACTGTTATGA	31	617
H31e6703PCR1	TATCTACTTGTGTGCTCTGT	31	766
H31e6704PCR2	TGACCTCCACTGTTATGAGCAATT	31	619
H31e6704PCR1	TGCGAATATCTACTTGTGTGCTCT GT	31	766
H31e6705PCR2	ACTGACCTCCACTGTTAT	31	617
H31e6705PCR1	CACGATTCCAAATGAGCCCAT	31	809
H33e6701PCR2	TATCCTGAACCAACTGACCTAT	33	618
H33e6701PCR1	TTGACACATAAACGAACTG	33	763
H33e6703PCR2	TCCTGAACCAACTGACCTAT	33	620
H33e6703PCR1	CCCATAAGTAGTTGCTGTAT	33	807
H33e6702PCR2	GACCTTTGTGTCCTCAAGAA	33	431
H33e6702PCR1	AGGTCAGTTGGTTCAGGATA	33	618
H35e6701PCR2	ATTACAGCGGAGTGAGGTAT	35	217
H35e6701PCR1	GTCTTTGCTTTTCAACTGGA	35	442
H35e6702PCR2	.TCAGAGGAGGAGGAAGATACTA	35	655
H35e6702PCR1	GATTATGCTCTCTGTGAACA	35	844
H35e6703PCR2	CCCGAGGCAACTGACCTATA	35	610
H35e6703PCR1	GTCAATGTGTGTGCTCTGTA	35	770
H52e6701PCR2	TTGTGTGAGGTGCTGGAAGAAT	52	144
H52e6701PCR1	CCCTCTCTTCTAATGTTT	52	358
H52e6702PCR2	GTGCCTACGCTTTTTATCTA	52	296
H52e6702PCR1	GGGGTCTCCAACACTCTGAACA	52	507
H58e6701PCR2	TCAGGCGTTGGAGACATC	58	157
H58e6701PCR1	AGCAATCGTAAGCACACT	58	301
H58e6702PCR2	TCTGTGCATGAAATCGAA	58	173
H58e6702PCR1	AGCACACTTTACATACTG	58	291
HBe6701PCR2	TACACTGCTGGACAACAT	B(11)	514
HBe6701PCR1	TCATCTTCTGAGCTGTCT	B(11)	619
HBe6702PCR2	TACACTGCTGGACAACATGCA	B(11)	514
HBe6702PCR1	GTCACATCCACAGCAACAGGTCA	B(11)	693
HBe6703PCR2	TGACCTGTTGCTGTGGATGTGA	B(11)	693
HBe6703PCR1	TACCTGAATCGTCCGCCAT	B(11)	832
HCe6701PCR2	CATGCCATAAATGTATAGA	C (18 39 45	295
HCe6701PCR1	CACCGCAGGCACCTTATTAA	C (18 39 45	408
H39e6701PCR2	GCAGACGACCACTACAGCAAA	39	210
H39e6701PCR1	ACACCGAGTCCGAGTAATA	39	344
H39e6702PCR2	TATTACTCGGACTCGGTGT	39	344
H39e6702PCR1	CTTGGGTTTCTCTTCGTGTTA	39	558
H39e6703PCR2	GAAATAGATGAACCCGACCA	39	703
H39e6703PCR1	GCACACCACGGACACACAAA	39	886
H45e6701PCR2	AACCATTGAACCCAGCAGAAA	45	430
H45e6701PCR1	TCTTTCTTGCCGTGCCTGGTCA	45	527
H45e6702PCR2	GAAACCATTGAACCCAGCAGAAAA	45	428
H45e6702PCR1	TTGCTATACTTGTGTTTCCCTACG	45	558

Primer name	Sequence	HPV type	nt
H45e6703PCR2	GTTGACCTGTTGTGTTACCAGCAAT	45	656
H45e6703PCR1	CACCACGGACACACAAAGGACAAG	45	868
H45e6704PCR2	CTGTTGACCTGTTGTGTTACGA	45	654
H45e6704PCR1	CCACGGACACACAAAGGACAAG	45	868
H45e6705PCR2	GTTGACCTGTTGTGTTACGA	45	656
H45e6705PCR1	ACGGACACACAAAGGACAAG	45	868
H51e6701PCR2	GGAGGAGGATGAAGTAGATA	51	658
H51e6701PCR1	GCCCATTAACATCTGCTGTA	51	807
H51e6702PCR2	AGAGGAGGAGGATGAAGTAGATA	51	655
H51e6702PCR1	ACGGGCAAACCAGGCTTAGT	51	829
H56e6701PCR2	TTGGGGTGCTGGAGACAAACATCT	56	519
H56e6701PCR1	TTCATCCTCATCCTCATCCTCTGA	56	665
H56e6702PCR2	TGGGGTGCTGGAGACAAACATC	56	520
H56e6702PCR1	CATCCTCATCCTCATCCTCTGA	56	665
H56e6703PCR2	TTGGGGTGCTGGAGACAAACAT	56	519
H56e6703PCR1	CCACAAACTTACACTCACAACA	56	764
H56e6704PCR2	GATTTTCCTTATGCAGTGTG	56	279
H56e6704PCR1	GACATCTGTAGCACCTTATT	56	410

Preferred PCR primer-pairs for HPV types 16, 18, 31 and 33 are analogous to the NASBA primer-pairs.

Table 5-Preferred L1 NASBA primers and probes

Primer name	Sequence
Onc2A2	5' GATGCAAGGTCGCATATGAGAATGGCATTTGTTGGGGTAA 3'
Onc2A1	5' AATTCTAATACGACTCACTATAGGGAGAAGGTCATATTCCTCCCCATGTC 3'
Onc2PoA	5' TTGTTACTGTTGTTGATACTAC 3'
Onc2B2	5' GATGCAAGGTCGCATATGAGAATGGCATTTGTTGGSRHAA 3'
Onc2B1	5' AATTCTAATACGACTCACTATAGGGAGAAGGTCATATTCCTCMMCATGDC 3'
Onc2PoB	5' TTGTTACTGTTGTTGATACYAC 3'
Onc2PoC	5' TTGTTACTGTTGTTGATACCAC 3'
Onc2C2	5' GATGCAAGGTCGCATATGAGAATGGCATTTGTTGGSIIAA 3'
Onc2D2	5' GATGCAAGGTCGCATATGAGAATGGCATTTGTTGGIIHAA 3'
Onc2E2	5' GATGCAAGGTCGCATATGAGAATGGCATTTGTTGGIRIAA 3'
Onc2F2	5' GATGCAAGGTCGCATATGAGAATGGCATTTGTTGGGGTAA 3'

Onc2G2	5' GATGCAAGGTCGCATATGAGAATGGCATTTGTTGGGGAAA 3'
Onc2H2	5' GATGCAAGGTCGCATATGAGAATGGCATTTGTTGGCATAA 3'
Onc2I2	5' GATGCAAGGTCGCATATGAGAATGGCATTTGTTGGGGCAA 3'
Onc2J2	5' GATGCAAGGTCGCATATGAGAATGGCATTTGTTGGCACAA 3'
Onc2K1	5' AATTCTAATACGACTCACTATAGGGAGAAGGTCATATTCCTCMICATGIC 3'
Onc2L1	5' AATTCTAATACGACTCACTATAGGGAGAAGGTCATATTCCTCAACATGIC 3'
Onc2M1	5' AATTCTAATACGACTCACTATAGGGAGAAGGTCATATTCCTCIICATGTC 3'
Onc2N1	5' AATTCTAATACGACTCACTATAGGGAGAAGGTCATATTCCTCIICATGGC 3'
Onc2O1	5' AATTCTAATACGACTCACTATAGGGAGAAGGTCATATTCCTCIICATGAC 3'
Onc2P1	5' AATTCTAATACGACTCACTATAGGGAGAAGGTCATATTCCTCIICATGCC 3'

Table 6-Preferred L1 PCR primers

Primer name	Sequence
Onc2A1-PCR	5' AATGGCATTTGTTGGGGTAA 3'
Onc2A2-PCR	5' TCATATTCCTCCCCATGTC 3'
Onc2B1-PCR	5' AATGGCATTTGTTGGSRHAA 3'
Onc2B2-PCR	5' TCATATTCCTCMMCATGDC 3'
Onc2C1-PCR	5' AATGGCATTTGTTGGSIIAA 3'
Onc2D1-PCR	5' AATGGCATTTGTTGGIIHAA 3'
Onc2E1-PCR	5' AATGGCATTTGTTGGIRIAA 3'
Onc2F1-PCR	5' AATGGCATTTGTTGGGGTAA 3'
Onc2G1-PCR	5' AATGGCATTTGTTGGGGAAA 3'
Onc2H1-PCR	5' AATGGCATTTGTTGGCATAA 3'
Onc2I1-PCR	5' AATGGCATTTGTTGGGGCAA 3'
Onc2J1-PCR	5' AATGGCATTTGTTGGCACAA 3'
Onc2K2-PCR	5' TCATATTCCTCMICATGIC 3'
Onc2L2-PCR	5' TCATATTCCTCAACATGIC 3'
Onc2M2-PCR	5' TCATATTCCTCIICATGTC 3'
Onc2N2-PCR	5' TCATATTCCTCIICATGGC 3'
Onc2O2-PCR	5' TCATATTCCTCIICATGAC 3'
Onc2P2-PCR	5' TCATATTCCTCIICATGCC 3'

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The HPV-specific sequences in SEQ ID NOs:149 and 150 (primers Onc2A2/Onc2A1-PCR and Onc2A1/Onc2A2-PCR) are identical to fragments of the HPV type 16 genomic sequence from position 6596-6615 (SEQ ID NO:149; Onc2A2/Onc2A1-PCR), and from position 6729 to 6747(SEQ ID NO:150; Onc2A1/Onc2A2-PCR).

The HPV-specific sequences SEQ ID NOs:152 and 153 (Onc2B2/Onc2B1-PCR and Onc2B1/Onc2B2-PCR) are variants of the above sequences, respectively, including several degenerate bases. Representations of the sequences of degenerate oligonucleotide molecules provided herein use the standard IUB code for mixed base sites: N=G,A,T,C; V=G,A,C; B=G,T,C; H=A,T,C; D=G,A,T; K=G,T; S=G,C;W=A,T; M=A,C; Y=C,T; R=A,G.

It is also possible to use variants of the HPV-specific sequences SEQ ID NO:152 (Onc2B2/Onc2B1-PCR) and SEQ ID NO:153 (Onc2B1/Onc2B2-PCR) wherein any two of nucleotides "SRH" towards the 3' end of the sequence are replaced with inosine (I), as follows:

5' AATGGCATTTGTTGGIIHAA 3'
5' AATGGCATTTGTTGGSIIAA 3'
5' AATGGCATTTGTTGGIRIAA 3'

The HPV-specific sequences SEQ ID NOs: 156-163 (present in primers Onc2C2, Onc2D2, Onc2E2, Onc2F2, Onc2G2, Onc2H2, Onc2I2, Onc2J2, Onc2C1-PCR, Onc2D1-PCR, Onc2E1-PCR, Onc2F1-PCR, Onc2G1-PCR, Onc2H1-PCR, Onc2I1-PCR and Onc2J1-PCR) are variants based on the HPV-specific sequence SEQ ID NO:152 (Onc2B2/Onc2B1-PCR), whereas the HPV-specific sequences SEQ ID NOs: 164-169 (present in primers Onc2K1, Onc2L1, Onc2M1, Onc2N1, Onc2O1, Onc2P1, Onc2K2-PCR, Onc2L2-PCR,

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Onc2M2-PCR, Onc2N2-PCR, Onc2O2-PCR and Onc2P2-PCR are variants based on the HPV-specific sequence SEQ ID NO:153 (Onc2B1/Onc2B2-PCR). These variants include degenerate bases and also inosine (I) residues. This
5   sequence variation enables oligonucleotides incorporating the variant sequences to bind to multiple HPV types. Inosine bases do not interfere with hybridization and so may be included at sites of variation between HPV types in order to construct a
10 "consensus" primer able to bind to multiple HPV types.

    Any one or more of primers Onc2A2, Onc2B2, Onc2C2, Onc2D2, Onc2E2, Onc2F2, Onc2G2, Onc2H2, Onc2I2 and Onc2J2, may be used in combination with any one or
15 more of primers Onc2A1, Onc2B1, Onc2K1, Onc2L1, Onc2M1, Onc2N1, Onc2O1 and Onc2P1, for NASBA amplification of HPV L1 mRNA.

    Any one or more of primers Onc2A1-PCR, Onc2B1-
20 PCR, Onc2C1-PCR, Onc2D1-PCR, Onc2E1-PCR, Onc2F1-PCR, Onc2G1-PCR, Onc2H1-PCR, Onc2I1-PCR and Onc2J1-PCR, may be used in combination with any one or more of primers Onc2A2-PCR, Onc2B2-PCR, Onc2K2-PCR, Onc2L2-PCR, Onc2M2-PCR, Onc2N2-PCR, Onc2O2-PCR and Onc2P2-PCR for
25 PCR amplification of HPV L1 mRNA.

    The invention will be further understood with reference to the following experimental examples and figures in which:
30   Figure 1A shows the results of a single reaction real-time NASBA assay using a FAM molecular beacon for HPV 16 on a patient sample while figure 1B shows multiplexed real-time NASBA assay using the FAM molecular beacon of Figure 1A and a molecular beacon
35 labeled with Texas red for UIA,

Figure 2A shows single reaction real-time NASBA with FAM molecular beacon for HPV 18 on a patient sample while figure 2B shows a multiplexed version with a Texas red labeled molecular beacon for HPV 18
5   and a FAM labeled beacon for HPV 33,

Figure 3A shows single reaction real-time NASBA with HPV 31 FAM labeled molecular beacon while figure 3B shows multiplexed version including HPV 45 Texas red labeled molecular beacon,

10   Figure 4A shows single reaction real-time NASBA with HPV 33 FAM labeled molecular beacon while figure 4B is a multiplexed version including a Texas red labeled HPV 18 molecular beacon,

Figure 5A shows single reaction real-time NASBA
15   with HPV45 FAM·labeled molecular beacon while figure 5B shows the multiplexed version including HPV 45 Texas red labeled molecular beacon and a FAM labeled HPV 31 molecular beacon, and

Figure 6 shows HPV detected by PreTect HPV-
20   Proofer and PCR compared to cytology or histology.

Example 1-Detection of HPV mRNA by NASBA-based nucleic acid amplification and real-time detection

25   Collection and preparation of clinical samples

Pap smears and HPV samples were collected from 5970 women in the cervical screening program in Oslo, Norway. Samples intended for RNA/DNA extraction were treated as follows:

30

Cervical samples were collected from each women attending the cervical screening program using a cytobrush(Rovers Medical Devices, The Netherlands). The cytobrush was then immersed in 9 ml lysis buffer
35   (5M Guanidine thiocyanate). Since RNA is best

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protected in the 5M guanidine thiocyanate at -70°C
only 1 ml of the total volume of sample was used for
each extraction round. The samples in lysis buffer
were stored at -20°C for no more than one week, then
5   at -70°C until isolation of DNA / RNA.

RNA and DNA were automatically isolated from 5300
women in the first round of extraction, using 1ml from
the total sample of 9ml in lysis buffer. RNA and DNA
10   were extracted according to the "Booms" isolation
method from Organon Teknika (Organon Teknika B.V.,
Boselind 15, P.O. Box 84, 5280 AB Baxtel, The
Netherlands; now Biomérieux, 69280 Marcy l'Etoile,
France) using the Nuclisens™ extractor following the
15   protocol for automated extraction.

Cell lines

      DNA and RNA from HeLa (HPV 18), SiHa (HPV 16) and
CaSki (HPV 16) cell lines were used as positive
20   controls for the PCR and NASBA reactions. These cells
were also used as sample material in the sensitivity
study (Example 2). SiHa cells have integrated 1 - 2
copies of HPV 16 per cell, whilst CaSki cells have
between 60-600 copies of HPV 16, both integrated and
25   in the episomal state. HeLa cells have approximately
10-50 copies of HPV 18 per cell.

HPV detection and typing by PCR

      Isolated DNA from cervical scrapes was subjected
30   to PCR using the consensus GP5+/6+ primers (EP-B-0 517
704). The PCR was carried out in 50 µl reaction
volume containing 75 mM Tris-HCl (pH 8.8 at 25°C), 20
mM $(NH_4)_2SO_4$, 0.01 % Tween 20™, 200 mM each of dNTP,
1.5 mM $MgCl_2$, 1 U recombinant Taq DNA Polymerase (MBI
35   Fermentas), 3 µl DNA sample and 50 pmol of each GP5+

and GP6+ primers. A 2 minutes denaturation step at
94°C was followed by 40 cycles of amplification with a
PCR processor (Primus 96, HPL block, MWG, Germany).
Each cycle included a denaturation step at 1 minutes,
5   a primer annealing step at 40°C for 2 minutes and a
chain elongation step at 72°C for 1.5 minutes. The
final elongation step was prolonged by 4 minutes to
ensure a complete extension of the amplified DNA.

10   The GP5+/6+ positive samples were subjected to
HPV type 16, 31 and 33 PCR protocols as follows:
HPV 16, 31 and 33: The PCR was carried out in 50 µl
containing 75 mM Tris-HCl (pH 8.8 at 25°C), 200 mM
each of dNTP, 1.5 mM MgCl$_2$, 2.5 U recombinant Taq DNA
15   Polymerase (MBI Fermentas), 3 µl DNA sample and 25
pmol of each primers. A 2 minutes denaturation step
at 94°C was followed by 35 cycles of amplification
with a PCR processor (Primus 96, HPL block, MWG,
Germany). Each cycle included a denaturation step at
20   30 sec, a primer annealing step at 57°C for 30 sec and
a chain elongation step at 72°C for 1 minutes. The
final elongation step was prolonged by 10 minutes to
ensure a complete extension of the amplified DNA. The
protocol for HPV 33 had a primer annealing step at
25   52°C. HPV 18 protocol: Primers were designed to
identify HPV type 18. The PCR was carried out in 50 µl
containing 75 mM Tris-HCl (pH 8,8 at 25°C), 20 mM
(NH$_4$)$_2$SO$_4$, 0.01 % Tween 20, 200 mM each of dNTP, 2.0 mM
MgCl$_2$, 2.5 U recombinant Taq DNA Polymerase (MBI
30   Fermentas), 3 µl DNA sample and 25 pmol of each
primer. A 2 minutes denaturation step at 94°C was
followed by 35 cycles of amplification in a PCR
processor (Primus 96, HPL block, MWG, Germany). Each
cycle included a denaturation step at 30 sec, a primer
35   annealing step at 57°C for 30 sec and a chain
elongation step at 72°C for 1 minutes. The final

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elongation step was prolonged by 10 minutes to ensure
a complete extension of the amplified DNA.

A primer set directed against the human β-globin
gene was used as a control of the DNA quality
5   (Operating procedure, University Hospital Vrije
Universiteit, Amsterdam, The Netherlands). The PCR was
carried out in 50 μl containing 75 mM Tris-HCl (pH 8.8
at 25°C), 200 mM each of dNTP, 1,5 mM MgCl$_2$, 1 U
Recombinant Taq DNA Polymerase (MBI Fermentas), 3 μl
10   DNA sample and 25 pmol of each primer. A 2 minutes
denaturation step at 94°C was followed by 35 cycles of
amplification with a PCR processor (Primus 96, HPL
block, MWG, Germany). Each cycle included a
denaturation step at 94°C for 1 minute, a primer
15   annealing step at 55°C for 1 ½ minutes and a chain
elongation step at 72°C for 2 minutes. The final
elongation step was prolonged by 4 minutes to ensure a
complete extension of the amplified DNA. HeLa was used
as positive controls for HPV 18, while SiHa or CaSki
20   were used as positive control for HPV 16. Water was
used as negative control.

Primers used for HPV PCR:

Type	Primer	Primer sequence	Position	Length (bp)
HPV16	Pr1	5' TCA AAA GCC ACT GTG TCC TGA 3'	421 – 440	119
	Pr2	5' CGT GTT CTT GAT GAT CTG CAA 3'	521 – 540	
HPV18	Pr1	(5' TTC CGG TTG ACC TTC TAT GT 3')	651 – 670	186
	Pr2	(5' GGT CGT CTG CTG AGC TTT CT 3')	817 – 836	
HPV31	Pr1	5' CTA CAG TAA GCA TTG TGC TAT GC 3'	3835 – 3875	153
	Pr2	5' ACG TAA TGG AGA GGT TGC AAT AAC CC 3'	3963 – 3988	
HPV33	Pr1	5' AAC GCC ATG AGA GGA CAC AAG 3'	567 – 587	211
	Pr2	5' ACA CAT AAA CGA ACT GTG TGT 3'	758 – 778	
Gp+	Gp5+	5' TTT GTT ACT GTG GTA GAT ACT AC 3'	6624 – 6649	150
	Gp6+	5' GAA AAA TAA ACT GTA AAT CAT ATT C	6719 – 6746	
BGPCO3	Pr1	5' ACA CAA CTG TGT TCA CTA GC		
BGPCO5	Pr2	5' GAA ACC CAA GAG TCT TCT CT		

Visualization of the PCR products was done on a DNA 500 chip (Agilent Technologies, USA) according to their manual. The DNA chip uses micro scale gel electrophoresis with an optimal detection limit of 0.5 -50 ng/ml. The results were interpreted using the Bioanalyzer 2100 software (Agilent Technologies, USA).

The following table confirms primers used for HPV PCR in patient samples and indicates additional PCR primers useful for HPV 35, 39, 45, 51, 52, 58 and HPV 6/11.

PCR primers for detection of HPV.

Type	Primer	Primer sequence	Position	Length (bp)
HPV 6/11	Pr1	5' TAC ACT GCT GGA CAA CAT 3'	514 – 531	123
	Pr2	5' TCA TCT TCT GAG CTG TCT 3'	619 - 636	
HPV16	Pr1	5' TCA AAA GCC ACT GTG TCC TGA 3'	421 – 441	120
	Pr2	5' CGT GTT CTT GAT GAT CTG CAA 3'	520 – 540	
HPV18	Pr1	5' TTC CGG TTG ACC TTC TAT GT 3'	651 – 670	186
	Pr2	5' GGT CGT CTG CTG AGC TTT CT 3'	817 – 836	
HPV31	Pr1	5' CTA CAG TAA GCA TTG TGC TAT GC 3'	3835 – 3857	155
	Pr2	5' ACG TAA TGG AGA GGT TGC AAT AAC CC 3'	3964 – 3989	
HPV33	Pr1	5' AAC GCC ATG AGA GGA CAC AAG 3'	567 – 587	212
	Pr2	5' ACA CAT AAA CGA ACT GTG GTG 3'	758 – 778	
HPV 35	Pr1	5' CCC GAG GCA ACT GAC CTA TA 3'	610-629	231
	Pr2	5' GGG GCA CAC TAT TCC AA ATG 3'	821-840	
HPV 39	Pr1	5' GCA GAC GAC CAC TAC AGC AAA 3'	210 – 230	153
	Pr2	5' ACA CCG AGT CCG AGT AAT A 3'	344 - 362	
HPV 45	Pr1	5' GAA ACC ATT GAA CCC AGC AGA AAA 3'	428 – 451	154
	Pr2	5' TTG CTA TAC TTG TGT TTC CCT ACG 3'	558 - 581	
HPV 51	Pr1	5' GGA GGA GGA TGA AGT AGA TA 3'	658 - 677	169
	Pr2	5' GCC CAT TAA CAT CTG CTG TA 3'	807 - 826	
HPV 52	Pr1	5' GTG CCT ACG CTT TTT ATC TA 3'	296 – 315	233
	Pr2	5' GGG GTC TCC AAC ACT CTG AAC A 3'	507 - 528	
HPV 58	Pr1	5' TCA GGC GTT GGA GAC ATC 3'	157 - 174	162
	Pr2	5' AGC AAT CGT AAG CAC ACT 3'	301 - 318	
Gp+	Gp5+	5' TTT GTT ACT GTG GTA GAT ACT AC 3'		150
	Gp6+	5' GAA AAA TAA ACT GTA AAT CAT ATT C		
BGPCO3	Pr1	5' ACA CAA CTG TGT TCA CTA GC		
BGPCO5	Pr2	5' GAA ACC CAA GAG TCT TCT CT		

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NASBA RNA amplification

Precautions for avoiding contamination:

1. Perform nucleic acid release, isolation and amplification/detection in separate laboratory areas.

5   2. Store and prepare reagents for nucleic acid release, isolation and amplification/detection at the laboratory areas where nucleic acid release, isolation and amplification/detection are to be performed, respectively.

10   3. Keep all tubes and vials closed when not in use.

4. Pipettes and other equipment that have been used in one laboratory area must not be used in the other areas.

5. Use a fresh pipette or pipette tip for each
15   pipetting action.

6. Use pipettes with aerosol resistant tips for fluids possibly containing nucleic acid. Pipetting of solutions must always be performed out of or into an isolated tube that is opened and closed exclusively
20   for this action. All other tubes and vials should be kept closed and separated from the one handled.

7. Use disposable gloves when working with clinical material possibly containing target-RNA or amplified material. If possible, change gloves after each
25   pipetting step in the test procedure, especially after contact with possibly contaminated material.

8. Collect used disposable material in a container. Close and remove container after each test run.

9. Soak tube racks used during nucleic acid isolation
30   or amplification/detection in a detergent (e.g. Merck Extran MA01 alkaline) for at least one hour after each test run.

The following procedure was carried out using reagents
35   from the Nuclisens™ Basic Kit, supplied by Organon

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Teknika.

Procedure for n=10 samples:-

5   1. Prepare enzyme solution.
       Add 55 µl of enzyme diluent (from Nuclisens™ Basic
       Kit; contains sorbitol in aqueous solution) to each of
       3 lyophilized enzyme spheres (from Nuclisens™ Basic
       Kit; contains AMV-RT, RNase H, T7 RNA polymerase and
10   BSA). Leave this enzyme solution at least for 20
       minutes at room temperature. Gather the enzyme
       solutions in one tube, mix well by flicking the tube
       with your finger, spin down briefly and use within 1
       hour. Final concentrations in the enzyme mix are 375
15   mM sorbitol, 2.5 µg BSA, 0.08 U RNase H, 32 U T7 RNA
       polymerase and 6.4 U AMV-reverse transcriptase.

       2. Prepare reagent sphere/KCl solution.
       For 10 samples: add 80 µl reagent sphere diluent (from
20   Nuclisens™ Basic Kit; contains Tris/HCl (pH 8.5), 45%
       DMSO) to the lyophilized reagent sphere (from
       Nuclisens™ Basic Kit; contains nucleotides,
       dithiotreitol and $MgCl_2$) and immediately vortex well.
       Do this with 3 reagent spheres and mix the solutions
25   in one tube.

       Add 3 µl NASBA water (from Nuclisens™ Basic Kit) to
       the reconstituted reagent sphere solution and mix
       well.
30
       Add 56 µl of KCl stock solution (from Nuclisens™ Basic
       Kit) and mix well. Use of this KCl/water mixture will
       result in NASBA reactions with a final KCl
       concentration of 70 mM. Final concentrations in the
35   reagent/KCl solution are 1 mM of each dNTP, 2 mM of

ATP, UTP and CTP, 1.5 mM GTP, and 0.5 mM ITP, 0.5 mM
dithiotreitol, 70 mM KCl, 12 mM $MgCl_2$, 40 mM Tris-HCl
(pH 8.5).

3. Prepare primer/probe solution containing target-
specific primers and molecular beacon probe.

For each target reaction transfer 91 µl of the reagent
sphere/KCl solution (prepared in step 2) into a fresh
tube. Add 25 µl of primers/molecular beacon probe
solution (to give final concentration of ~0.1-0.5 µM
each of the sense and antisense primers and ~ 15-70
pmol molecular beacon probe per reaction). Mix well
by vortexing. Do not centrifuge.

In case less than 10 target RNA amplifications are
being performed refer to the table below for the
appropriate amounts of reagent sphere solution,
KCl/water solution and primers to be used. Primer
solutions should be used within 30 minutes after
preparation.

Reactions (n)	Reagent sphere solution (µl)	KCl/water (µl)	Primer mix (µl)
10	80	30	10
9	72	27	9
8	64	24	8
7	56	21	7
6	48	18	6
5	40	15	5
4	32	12	4
3	24	9	3
2	16	6	2
1	8	3	1

4. Addition of samples

For each target RNA reaction:

In a 96 well microtiter plate pipette 10 µl of the
primer/probe solution (prepared in step 3) into each

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of 10 wells. Add 5 μl nucleic acid extract to each
well. Incubate the microtiter plate for 4 minutes at
65 ± 1 °C. Cool to at 41 ± 0.5 °C for 4 minutes.
Then to each well add 5 μl enzyme solution.

5   Immediately place the microtiter plate in a
fluorescent detection instrument (e.g. NucliSens™
EasyQ Analyzer) and start the amplification.

Results from clinical study

10   Table 7 shows the distribution of real-time NASBA
HPV positive (L1 and/or E6 expression) and PCR HPV
positive cases related to cytology results. PCR
amplification was carried out as described by Karlsen
et al., J Clin Microbiol. 34: 2095-2100, 1996. The

15   figures for expected histology are based on average
results from similar study on CIN III lesions (Clavel
et al., Br J Cancer, 84: 1616-1623, 2001). The
results from several example cases are listed in Table
8.

20

Table 7:

	Normal	Benign	Condyloma	CIN III
Cytology	4474	66	16	15
PCR	9.0%	44.6%	87.5%	73.3%
Real-time NASBA	1%	24.6%	37.5%	73.3%
Expected Histology	0.2%	5-15%	15-20%	71%

30

Table 8:

Internal No.	Cytology	PCR	L1 NASBA	E6 NASBA
84	Neg	Neg	Neg	31

289	Neg	31	Pos	31
926	Neg	Neg	Pos	16
743	Benign	Neg	Neg	33
1512	Benign	16	Pos	16
3437	Benign	Neg	Neg	18
3696	Benign	16	Pos	Neg
2043	Condyloma	16, 51	Pos	16
3873	Condyloma	16, 51	Pos	16
3634	CIN II	33	Neg	33
4276	CIN III	Neg	Neg	18
4767	CIN III	18	Neg	18
1482	CIN III	Neg	Pos	16
5217	CIN III	31	Neg	31
4696	CIN III	Neg	Neg	Neg

Example 2-Sensitivity of real-time NASBA on control cell lines

Cervical cancer cell lines, CaSki, SiHa and HeLa were diluted in lysis buffer either before automated extraction of nucleic acids using the Boom's extraction method from Organon Teknika/bioMerieux (parallels 1 and 3), or after nucleic acid extraction (parallel 2). Real-time NASBA was performed using molecular beacons probes labelled with Texas red (16, L1 and 18) or FAM (U1A, 33 and 31) following the protocol described above.

Table 9:

Primer sets and probes	CaSki						CaSki						HeLa					
	16 E6	U1	16 E6	U1	16 E6	U1	L1	33 E6	L1	33 E6	L1	33 E6	18 E6	31 E6	18 E6	31 E6	18 E6	31 E6
Parallels	1	1	2	2	3	3	1	1	2	2	3	3	1	1	2	2	3	3
Number of Cells																		
100 000	+	+	+	+	+	+	+	-	+	-	+	-	+	-	+	-	+	-
10 000	+	+	+	+	+	+	+	-	+	-	+	-	+	-	+	-	+	-
1 000	+	+	+	+	+	+	+	-	+	-	+	-	+	-	+	-	+	-
100	+	+	+	+	+	+	+	-	+	-	+	-	+	-	+	-	+	-
10	+	+	+	+	+	+	-	-	-	-	+	-	+	-	+	-	+	-
1	-	-	+	-	+	-	-	-	-	-	-	-	+	-	+	-	+	-
10^{-1}	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Thus, it is possible to detect HPV E6 mRNA in less than 1 cell using real-time NASBA.

Real-time NASBA was tested both as a multiplex assay and as single reactions. The results from the following sensitivity study are based on parallel runs of CaSki, SiHa and HeLa cell lines, and on three parallel runs on synthetic DNA oligos for HPV type 16, 18, 31 and 33. The definition of the detection limit is that both of the samples in the parallel are positive. The number in the brackets (x) indicates that the specified amount of cells also have been detected in some runs. Sensitivity is defined as the amount of cells necessary for detection of HPV in two parallel runs. The HPV types are determined from PCR and the specificity is based on NASBA compared to PCR.

Sensitivity

PCR: the HPV consensus PCR using Gp5+/6+ detected only down to 10^4 SiHa and HeLa cells, and down to 10^3 CaSki cells. However, the type specific PCR primer-sets were more sensitive, detecting 10^3 (10^2) SiHa cells and 0.1 CaSki cells for HPV 16 type specific PCR primer-set, while the HPV 18 type specific PCR primer-set detected

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10^2 HeLa cells.

Real-time NASBA: Real-time NASBA with primers specific
for U1A, detected 10(1) SiHa and CaSki cells and 1
HeLa cell in the reaction mixture. For the HPV 16
specific primers, the lower detection limit was (10)
(10^2, 1) SiHa cells and 10 (1) CaSki cells and for the
HPV 18 specific primers the detection limit was 1
(0.1) HeLa cell. The universal L1 primers detected 10
CaSki cells. HeLa cells and SiHa cells were not
detected with the universal L1 primers.

Real-time multiplex NASBA with the U1A specific
primers, had a lower detection limit of 10^2(10) SiHa
cells and 10(1) CaSki cells when combined with the HPV
16 specific primers, which had a lower detection limit
for 10(1) SiHa and 10(1) CaSki cells. The L1 specific
primers in combination with the HPV 33 specific
primers detected 10^3(102) CaSki cells. There was no
competing HPV 33 sample in the reaction. For the HPV
18 specific primers, the lower detection limit was 1
(0.1) HeLa cell when combined with the HPV 31 specific
primers. There was no competing HPV 31 sample in the
reaction. Sensitivity of the HPV 31 and HPV 33
specific primers were not tested, due to lack of cell
lines harbouring these HPV types. They were tested
against samples containing HPV 31 and HPV 33, but the
amount of cells and the copy number of HPV 31 and HPV
33 in these cells were unknown and most probably
varied in different samples.

Table 10: sensitivity of real-time NASBA compared to PCR

Primer	NASBA			PCR		
	SiHa	CaSki	HeLa	SiHa	CaSki	HeLa
GP5+/6+	-	-	-	10^4	10^3	10^3
L1	-	10^3 (10^2)	-	-	-	-
U1A	10^2 (10)	10 (1)	-	-	-	-
HPV 16	10 (1)	10	-	10^3 (10^2)	0.1	-
HPV 18	-	-	1 (0,1)	-	-	10^2

Real-time NASBA was performed on samples from women admitted to Østfold Central Hospital for treatment of CIN in the period of 1999-2001 (see example 3). Molecular beacon probes labeled with FAM or Texas red were used together with the nucleic acid extraction and NASBA protocols described above. The results are shown in figures 1A and 1B (HPV 16 - patient sample 205), figures 2A and 2B (HPV 18 - patient sample 146), figures 3A and 3B (HPV 31 - patient sample 236), figures 4A and 4B (HPV 33 - patient sample 218) and figures 5A and 5B (HPV 45 - patient sample 343). In each case, the "A" figure is a single reaction while the "B" figure is the multiplex assay.

Specificity: Cross reactivity of Real-time NASBA. Real-time NASBA primer combinations were tested against 490 cervical samples from the Oslo study positive with PCR for HPV 6/11, 16, 18, 31, 33, 35, 39, 45, 51, 52, 58 or HPV X to check for cross reactivity between HPV types using NASBA. All samples have been typed by consensus PCR and type specific PCR for the respective HPV types, except for HPV type 39(2), 52(1) and 58(2). These samples are added to test against PreTect HPV-Proofer. HPV X are positive for consensus Gp5+/6+ PCR but negative for HPV6/11, 16, 18, 31, 33, 35, 45 and 51 by type specific PCR. Results are shown in table 14. No cross-reactivity was shown. Sequence confirmation of a selected number

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of cases from table 14 is shown in table 14a.

PCR: a total of 773 cervical samples were tested with
PCR and the PreTect HPV-Proofer (Real time multiplex
5   NASBA), and a total of 24.6% (190/773) samples were
positive with the Gp5+/6+ consensus PCR primers. 74.1%
(83/112) were typed to be HPV 16, 13% (15/112) HPV 18,
17% (19/112) HPV 31 and 12% (13/112) HPV 33 including
multiple HPV infections. A total of 103 samples had
10   single or multiple HPV infections, and 91.3% (94/103)
had only a single HPV infection. Double HPV infections
occurred in 8.7% (9/103) of the samples. All samples
were first tested with the consensus Gp5+/6+ PCR
primers. The HPV PCR negative samples from the
15   consensus Gp5+/6+ were then tested with β-globin
control primers for a verification of intact DNA. The
HPV PCR positive samples were not subjected to this
DNA control. The HPV negative samples in this study
were all positive with β-globin control PCR primers.
20   Only DNA samples positive with Gp5+/6+ PCR were
subjected to HPV type specific PCR. HPV types of
interest were HPV 16, 18, 31 and 33.

Real-time multiplex NASBA: For the real-time NASBA
25   reactions, the primers and probes for the U1A gene
product were used as a performance control for intact
RNA. Samples negative for U1A were rejected.
A total of 14.2% (110/773) of the samples were
positive with at least one of the HPV type-specific
30   NASBA primers including samples showing multiple HPV
infections. From these samples 54.5% (60/110) were
positive with HPV 16 NASBA primers, 13.6% (15/110)
with HPV 18 primers, 21.8% (24/110) with HPV 31
primers and 13.6% (15/110) with HPV 33 primers. A
35   total of 45 samples were positive with the L1
consensus primers and usually together with HPV 16

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E6/E7 oncogene expression 82.2% (37/45). The consensus
L1 was detected in 2.2% (1/45) together with either
HPV 18, 31 and 33 respectively. L1 was also detected
alone in 8.9% (4/45) cases, and they all were PCR
5   positive with Gp5+/6+ primers. A total of 108 samples
had single or multiple HPV infections, and 98.1%
(106/108) had only a single HPV infections. Double
mRNA expression occurred in 1.9% (2/108) of the
samples.

10

Real-time multiplex NASBA compared to PCR: a total of
87 samples showed presence of HPV 16 DNA or RNA with
HPV 16 PCR or PreTect HPV-Proofer. 64.4% (56/87) were
determined to be positive for HPV 16 with both PCR and
15   real-time NASBA. 39.1% (34/87) were only positive with
PCR and 3.4% (3/87) were positive only with real-time
NASBA. For HPV 18, a total of 20 samples showed
presence of HPV 18 DNA or RNA with either PCR or real-
time NASBA. From these 20 samples, 50% (10/20) were
20   positive with both tests, and 35% (7/20) were only
positive with PCR and 15% (3/20) were only positive
with real-time NASBA. A total of 27 samples showed
presence of HPV 31 DNA or RNA with either PCR or real-
time NASBA. Out of these 27 samples, 59.3% (16/27)
25   were positive with both tests and 11.1% (3/27) were
positive only with the PCR test and 18.5% (5/27) were
only positive with the real-time NASBA test. For HPV
33, a total of 18 samples showed presence of HPV DNA
or RNA with either PCR or PreTect-HPV Proofer and
30   55.6% (10/18) of the samples were tested positive with
both tests. 16.7% (3/18) were only positive with PCR
and 22.2% (4/18) were only positive with real-time
NASBA.

35

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Table 11: statistical distribution of HPV in samples
with PCR and real-time NASBA

	PCR	%	NASBA	%
Total samples	773		773	
Total positive samples	190	24.6	110	14.2
HPV16	83	74.1	60	54.5
HPV 18	15	13	15	13.6
HPV 31	19	17	24	21.8
HPV33	13	12	15	13.6
HPV X	78	69.6	-	-

Table 12: correspondence between PCR and real-time
NASBA

	Total	Both tests	%	Only PCR +	% (PCR)	% (Total)	Only NASBA	% NASBA	% (total)
HPV 16	87	56	64.4	34	41.0	39.1	3	5	3.4
HPV 18	20	10	50.0	7	46.7	35.0	3	20	15.0
HPV 31	27	16	59.3	3	15.8	11.1	5	20.8	18.5
HPV 33	18	10	55.6	3	23.1	16.7	4	26.7	22.2

Table 13: Real-time NASBA results for L1

	Total	%
L1 (NASBA)	45	100
L1 + HPV 16	37	82.2
L1 alone	4	8.9
L1 + HPV 18	1	2.2
L1 + HPV 31	1	2.2
L1 + HPV 33	1	2.2

Table 14: Genetic specificity of real-time multiplex NASBA compared to PCR

NASBA Primers	HPV (PCR)												Total Number
	6/11	16	18	31	33	35	39	45	51	52	58	X	
16	2	28	1	0	1	1	0	0	5	0	0	2	
18	1	1	18	0	1	0	0	0	1	0	0	1	
31	1	0	1	13	1	5	0	1	1	0	0	0	
33	1	2	0	2	12	2	0	1	2	0	0	1	
45	2	1	1	1	1	1	0	17	1	0	0	1	
Sum tested	43	71	36	32	25	23	2	23	31	1	2	201	490

Table 14a: DNA sequencing from Gp5+ PCR primers (not to be included in the article)

Int No	HPV type by PCR	HPV type by PreTect HPV-Proofer	HPV type by Sequencing (BLAST)
1272	16	16	16
152	35		35
2655	58		58
2924	33	33	33
2942	18	18	18
2987	16	16	16
3016	33	33	33
3041	35		35
3393	35		35
3873	16	16	16
4767	18	18	18
5707	18	18	18
845	X		39

Discussion

Sensitivity of real-time NASBA was generally better than the sensitivity of PCR. The general sensitivity of real-time NASBA for all the markers were between 1 and 10^2 cells, which is considerable better than for the PCR reaction with a sensitivity range from 10^2 to 10^4. As expected, the sensitivity of the specific primers and probes were better than the

sensitivity of the universal primers and probes.
Real-time NASBA was just as sensitive or more
sensitive than real-time multiplex NASBA.

5   Real-time NASBA primers and molecular beacon
probe directed towards U1A (a human house keeping
gene) were used as a performance control of the sample
material in the real-time NASBA reaction to ensure
that the RNA in the sample material was intact. A
10   positive signal from this reaction was necessary for a
validation of the real-time NASBA reaction.

The sensitivity of the universal real-time NASBA
with L1 (the major capsid protein of HPV) was much
15   better than for the universal Gp5+/6+ PCR, also
directed against L1, with a sensitivity of 10 cells
compared to 10^3 (10^2) CaSki cells. These two primer
sets (PCR and NASBA) have their targets in the same
region of the conserved L1 gene of different HPV
20   types. The differences in sensitivity may be due to
the fact that there is usually one copy of each gene
per cell, while the copy number of mRNA may be several
hundreds. The real-time NASBA L1 primers did not
detect SiHa or HeLa cells as the Gp5+/6+ PCR primers
25   did, indicating lack of L1 expression in these cell
lines. Gp5+/6+ PCR primers detected 10^4 SiHa or HeLa
cells. Considering the amount of HPV copies in each
cell, it makes sense that the CaSki cells were
detected in 1/10 the amount of cells from SiHa and
30   HeLa since CaSki cells have 60-600 HPV copies per
cell, both integrated and episomal, while SiHa cells
have 1-2 HPV copies integrated per cell and HeLa cells
have 10-50 HPV copies integrated per cell. The L1
primer set detected only CaSki cells, with both
35   integrated and episomal forms of HPV, and not in SiHa
or HeLa cells, with only integrated forms of HPV. This

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might indicate that the L1 gene is only expressed in
episomal states of HPV infection, and therefore L1 may
be a valuable marker for integration and persistence
of HPV infection.

5

The HPV type-specific NASBA primers are directed
against the full length E6/E7 transcript, which are
expressed in large amount in cancer cells due to lack
of E2 gene product. The real-time NASBA 16 type

10   specific primers detected 10(1) SiHa cells and 10(1)
CaSki cells compared to HPV 16 PCR primers that
detected $10^3(10^2)$ SiHa cells. The explanation for this
might be the different amount of HPV copies in each
cell line. The CaSki cells have both integrated and

15   episomal forms of HPV, while SiHa has only integrated
forms of HPV. This may be due to high expression of
mRNA from the E6/E7 genes. For detection of CaSki
cells, the detection limit for the NASBA HPV 16
primers were 10(1) CaSki cells compared to 0.1 CaSki

20   cells for the HPV 16 PCR primers. This is peculiar,
but an explanation may be that the CaSki cells contain
from 60-600 copies of HPV 16 DNA, so that it is
possible to detect 0.1 CaSki cells with 6-60 HPV 16
DNA copies. The lower sensitivity of real-time NASBA

25   compared to PCR may indicate that the expression of
E6/E7 in the CaSki cells is moderate/low. Degradation
of the unstable mRNA may also be an explanation. The
amount of HPV copies in the CaSki cells may be in the
order of 60-600 times more than in the SiHa cells,

30   which is shown by the more sensitive detection of
CaSki cells.

The type specific HPV 18 PCR primers detected 10^2
HeLa cells. This is a magnitude of 100 better than the

35   HPV consensus Gp5+/6+ primers and states that specific
primers are generally more sensitive than consensus

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primers. The sensitivity of the type specific HPV 18
NASBA primers was 1 (0,1) HeLa cells, indicating high
expression of E6/E7 in HeLa cells.

5   The sensitivity of U1A NASBA primers was 10 SiHa
or CaSki. The target for the U1A primer set is a human
housekeeping gene that is expressed in every human
cell.

10   The sensitivity of PCR and NASBA varies for
different primer sets and sample material, and
generally type specific primers are more sensitive
than consensus primers due to base pair mismatch in
consensus primer sets. The annealing temperature for
15   the primers in·the PCR reaction can be optimised,
giving optimal reaction condition for the primers. In
contrast to the annealing temperature in PCR, the
annealing temperature for the NASBA primers must be
fixed at 41°C. This lack of temperature flexibility
20   may make the NASBA primers less sensitive and specific
than the PCR primers.

           PCR amplifies double stranded DNA and the target
is usually present as one copy per cell and this makes
25   .it vulnerable to the number of cells in the sample
material. The target for the NASBA reaction is RNA,
and mRNA may be present as multiple copies per cell,
depending on the expression of the genes. By choosing
a gene that is highly expressed, the mRNA copy number
30   may be several hundred per cell and therefore easier
to detect.

           dsDNA is relatively stable in the cell and the
material stays intact for a long time. In contrast to
35   dsDNA, mRNA is generally not very stable and

degradation of mRNA is rapid depending on the cell.
There is no detected DNase or RNase activity in the
lysis buffer so both dsDNA and ssRNA should be stable.
Autocatalytic activity may degrade both DNA and RNA.

5   The DNA/RNA from the cervix sample should stay intact,
when stored in the lysis buffer, for 24 hours at
15-30°C, 7 days at 2-8°C or at -70°C for long term
storage.

10   A limitation in the real-time NASBA reaction is
the concentration of the molecular beacon probes. The
amount of products will exceed the concentration of
the molecular beacon probes and therefore it will not
be detected because a high molecular beacon probe

15   concentration will make the reaction mixture more
complex and inhibit the amplification reaction.
Nucleotides may also be a limitation to the final
amount of the amplification product, both in the PCR
and in the NASBA reaction. The final concentration of

20   the amplified product may in itself inhibit further
amplification because of the amount of product and the
complexity of the reaction mixture. During a NASBA
reaction in the presence of molecular beacons, the
probe might compete with the amplification by

25   hybridising to the template, making it unavailable for
following RNA synthesis. In this way, RNA is
subtracted as substrate for the reverse transcription
steps and further RNA synthesis by T7 RNA polymerase.
This competition is not significant with low amounts

30   of molecular beacon, and with a high amount of
molecular beacon this inhibition can be overcome by a
higher number of copies of input RNA.

       The linear relationship between the amount of
35   input RNA and the time-to-positive signal was tested
in a ten-fold serial dilutions of different HPV cell

lines. There was a clear indication that a positive
signal was dependent on the amount of input RNA and
time. The multiplex reaction needed more time than the
single reaction to show a positive signal. This might

5   be due to competition in the more complex mixture in
the multiplex reaction vessel and also to the fact
that the multiplex reaction has a different and lower
concentration of primer and probe. The relationship
between amount of target RNA and time to positive

10   signal opens up for a real-time multiplex quantitative
amplification reaction with internal RNA standards in
each reaction vessel.

Real-time NASBA: single vs. multiplex. Real-time NASBA

15   was generally more sensitive than real-time multiplex
NASBA. This was as expected because of competition
between primers and probes in the multiplex reaction.
The final concentration of primers and molecular
beacon probes were optimised in the multiplex reaction

20   so that for at least one of the primer and probe sets
the concentration were lower than in the single
reaction. From this it follows that with a lower
concentration of primers, the less sensitive the
reaction, or at least the less rapid the reaction. It

25   will take longer time to reach the exponential stage
of the amplification reaction and therefore longer
time to detect the products. The concentration of the
primers will not be a limitation to the final
concentration of the product in the NASBA reaction

30   because the double stranded DNA created from the
primers will continue to serve as a template for the
RNA polymerase over and over again in a loop. The
sensitivity of multiplex real-time NASBA was the same
for HPV 16 and HPV 18 compared to single real-time

35   NASBA, but the sensitivity for L1 decreased
drastically from a detection limit of 10(1) in the

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single reaction to $10^3(10^2)$ CaSki cells in the
multiplex reaction. For U1A NASBA primers, the
sensitivity decreased from 10(1) to $10^2(10)$ SiHa
cells, while the detection limit remained the same for

5   the CaSki cells. This decrease in detection limit may
be to more complex competition of primers and
molecular beacon probes in the multiplex reaction. The
final concentration of primers and molecular beacon
probes may not be the best and the different primers

10   and molecular beacon probes in the multiplex reaction
may interfere with each other. The U1A NASBA primers
detected 1 HeLa cell. One might expect the same
detection level in all the cell lines, but the
sensitivity of HeLa cells were 1/10 of the detection

15   level of SiHa and CaSki cells. These cell lines are
cancer cells and they might have different impact on
the cells so that the expression of U1A is different.
The differences may also be due to different amount of
cells in each reaction, because of counting errors

20   during harvesting of the cells.

       Real-time NASBA showed no cross reactivity
between HPV 16, 18, 31 and 33 or with HPV 6/11, 35,
39, 45, 51, 52, 58 or HPV X.

25
       The specificity of the PCR reaction may be better
than the specificity of the real-time NASBA reaction
because the NASBA reaction is an isothermal reaction
at 41°C with no possibilities to change the annealing

30   temperature of the primers. The primers are basically
designed the same way as for the PCR primers. In a PCR
reaction, you have the possibility to change the
annealing temperature, in contrast to the NASBA
reaction, and therefore choose an annealing

35   temperature that is optimal for the two primers. This
makes the annealing of the primers more specific. The

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PCR results where visualized with gel electrophoresis.
But the molecular beacon probes in the real-time NASBA
reaction is an additional parameter compared to PCR
and therefore may give the overall NASBA reaction a
5   better specificity. It is also easier to find two
different regions on the DNA sequence for primer
annealing because there is much greater flexibility in
the length of the PCR product, than for the NASBA
product, which should be less than 250 bp. It is
10   important for the specificity of the NASBA reaction to
choose a unique area that is not conserved among the
different HPV types. A couple of base pair mismatches
may still give an amplification or hybridisation of
the target.

15

Detection of CaSki (integrated and episomal
state) cells with the universal L1 NASBA primers and
not SiHa or HeLa (both integrated) may give an
indication that integrated HPV doesn't show any L1
20   expression, while HPV in the episomal state may have
L1 expression.

In summary, an identification assay has been
developed for HPV type 16, 18, 31 and 33 that can
25   accurately identify the oncogenic E6/E7 expression of
these HPV types. The assay can also identify the
expression of the major capsid protein, L1.

Example 3-Further clinical study in 190 patients

30

Patients/Clinical samples
Biopsies from 190 women admitted to Østfold
central-hospital for treatment of CIN in the period
1999-2001. The mean age of the 190 women included in
35   the study was 37.4 years (range 22-74 years). Biopsies

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were frozen in -80°C immediately after collection.

Cytological examination of samples

The routine cytological reports were used to record
cytological findings. No attempt was made to
re-evaluate the slides. Each one of them indicated a
CIN II-III condition, i.e. a high grade dysplasia or
HSIL, which was the basis for hospital admittance,
colposcopy and biopsy.

Histological examination of samples

A biopsy, here termed *biopsy 1*, was taken after a
high-grade cytology report. If it confirmed a
high-grade lesion (CIN II or III), the patient was
again admitted to hospital, this time for
colposcopically guided conization. Before the
conization, but after local anesthesia was applied, a
second biopsy (*biopsy 2*) was taken from an area of
portio where a dysplasia was most likely to be
localised, judged from the gross findings. This biopsy
(2 x 2 mm) was frozen within 2 minutes in a -80°C
freezer.

Biopsy 2 was split in two when frozen and half
was used for DNA/RNA extraction. The other half was
fixed in 10% buffered formaldehyde and processed for
histopathological examination. Some lesions were not
correctly oriented in the paraffin block and had to be
reoriented or serial sectioned in order to show the
relevant surface epithelium. Consequently, it cannot
be guaranteed that exactly the same tissue was used
for the extraction and for the histopathological
evaluation. The cone specimen, finally, was evaluated
by the local pathologist, who in all cases could
confirm the presence of dysplasia. It was not always

the same grade as in the original biopsy, and, in many
cases, not the same as in *biopsy 2*.

Extraction of nucleic acids

5   Nucleic acids were isolated using the automated
Nuclisens Extractor as previously described (Boom et
al., 1990). Each biopsy was cut in two pieces, one
intended for histological examination and the other
half for RNA analysis. The material intended for RNA
10   analysis was divided into smaller pieces while kept on
dry ice (-80°C) and put into 1 ml of lysisbuffer (as
above) followed by 20 seconds of homogenisation using
disposable pestles. 100 ml of the sample was further
diluted 10 fold in lysisbuffer and 100 ml was then
15   extracted for DNA/RNA. The extracted DNA/RNA was
eluted with ~40 ml of elution buffer (Organon Teknika)
and stored at -70°C.

       All molecular beacon probes used in this study
20   employ the fluorophore FAM (6-carboxyfluorescein) at
the 5'end of the structure. This was bound to a
variable stem-loop sequence coupled to the universal
quencher 4-(4'dimethylaminophenylazo)benzoic acid
(DABCYL) at the 3'end. The probes were delivered by
25   Eurogentec, Belgium. Final concentration of MBs used
in the reaction was 2.5 mM. For the real-time NASBA we
made use of the NucliSens Basic Kit (Organon Teknika,
Netherlands), intended for the development of
user-defined RNA amplification assays. The NASBA
30   amplification was carried out in a volume of 20 µl.
The primer-sets and probes were directed against
full-length E6/E7 mRNA for the high-risk HPV 16,18,
31, and 33. As performance control, to avoid false
negative results due to degradation of nucleic acid,
35   we used a primer set and probe directed against the
human U1 small nuclear ribonucleoprotein (snRNP)

specific A protein (U1A mRNA) (Nelissen et al., 1991).
All samples were run in duplicate on separate machines
(microplate readers for measuring fluorescence and
absorbance, Bio-tek FL-600 FA from MWG). mRNA

5   isolated from CaSki/SiHa or HeLa cells served as
positive controls for HPV 16 and HPV 18 transcripts,
respectively. Negative controls, included for every 7
reaction, consisted of a reaction containing all
reagents except mRNA.

10

HPV DNA analysis; Polymerase Chain Reaction
       The same extracts and amounts as used in the
NASBA reaction were used for PCR. The L1 consensus
primers Gp5+/Gp6+ were used to detect all samples

15   containing HPV DNA. The PCR amplification was carried
out as described above. The first DNA denaturation
was done for 2 minutes at 94°C, then 40 cycles of PCR
were run: denaturation 1 minute at 94°C, annealing for
2 minutes at 40°C, extension for 1.5 minutes at 72°C,

20   followed by a final extension for 4 minutes at 72°C.
Typing of HPV was performed by using PCR type-specific
primers against HPV 16, 18, 31, and 33 (6/11, 35, 45,
51, 52, 58), as described above.

25   Results
       Originally 190 patients were biopsied after being
given the diagnosis CIN I, CINII, or CIN III by
cytology. A high-grade lesion was confirmed by
histologically examination, 150 samples diagnosed as

30   CIN III (78.9%). Biopsy 2, taken before conization,
was used for RNA analysis. However, histological
examination of this biopsy diagnosed only 53 samples
of the originally 150 as CIN III [54 were given no
diagnosis, 24 diagnosed as CIN II, 18 as CIN I, and 4

35   as HPV/condylom]. The number of CIN II samples
increased from 16 (8,4%) to 30 (15,8%) [by Histology I

24 diagnosed as CIN III, 4 as CIN II, 1 as carcinom,
and 1 as CIN I. 12 CIN II cases from Histology I were
given a lower diagnosis in Histology II]. The degree
of CIN I increased from 6 samples (3.2%) to 32 samples

5   (16.8%). The 2 squamous cell carcinomas were in
Histology II diagnosed as CIN III, the adenocarcinom
as CIN II. In 71 samples (38.4%) high-grade lesions
were not detected.

10   HPV oncogenic RNA was detected in 69 (36%) of the
190 patients. Of the 53 samples (28%) diagnosed as CIN
III in Histology II, we found 40 (76%) cases showing
HPV 16, 18, 31, or 33 oncogenic expression. In
addition, we found oncogenic expression in 9 of 30

15   cases (30%) of CIN II, in 4 of 32 cases (13%) of CIN
I, in 14 of 71 cases (20%) not showing cell
abnormalities, and in 2 of 4 (50%) samples diagnosed
as HPV/condyloma.

20   HPV 16 RNA was found in 42 of the 190 patients,
HPV 18 was found in 7 (3.7%), HPV 31 in 15 (7.9%), and
HPV 33 in 8 (4.2%). One patient had mixed infection
with HPV 16 and HPV 18, and one with HPV 16 and HPV
31.

25
        Using the consensus Gp5+/Gp6+ primers directed
against the L1 gene, encoding the major capsid
protein, PCR detected HPV in 81 of the 190 cervical
biopsies (43%). Of the 119 cases given a diagnosis in

30   the second histological examination (115 diagnosed as
CIN, 4 as HPV/condyloma) 63 were found to contain HPV
DNA. The additional 18 cases detected were not given
any histological diagnosis. 20 of the 81 cases were
not detected by NASBA; 7 out of these were given the

35   diagnosis CIN III, 2 were diagnosed as CIN II, 4
diagnosed as CIN I, and 7 given no diagnosis.

Type-specific PCR detected 85 cases containing
HPV; 66 having HPV 16, 10 HPV 18, 14 HPV 31, 7 HPV 33.
12 cases had multiple infection: 3 with HPV 16+18; 4
with HPV 16+33, 5 with HPV 16+31. 20 no diagnosis.

5

Example 4

HPV detected by PreTect HPV-Proofer and PCR compared
to cytology and histology:

10   Normal and ASCUS samples (including borderline
smears) were determined by cytology. All samples were
tested with consensus PCR and PreTect HPV-Proofer but
only the consensus positive samples were typed by PCR.
The CIN 3 and cancer samples were determined by

15   histology and all the samples were tested with all
three methods. The results are shown in Figure 6.
Concordance between real-time multiplex NASBA and PCR
compared to cytology or histology is shown in Table 15
below.

20

Table 15: Concordance between real-time multiplex NASBA
and PCR compared to cytology or histology

Cytology/Histology	Concordance[a] (Number)	Concordance[b] (Number)
Normal	98.2% (4043)	42.8% (138)
ASCUS[c]	94.5% (55)	78.6% (14)
CIN 3	94.3% (53)	93.2% (44)
Cancer	99.0% (196)	98.8% (170)

30   Only samples positive by Gp5+/6+ PCR have been typed.
[a] Including PCR and real-time multiplex NASBA positive
and negative samples. [b] Including only PCR and / or real-
time multiplex NASBA positive samples. [c] ASCUS excluding
borderline smears.

35

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Example 5

The invention provides a kit for detection of mRNA
transcripts from the E6 gene(s) of HPV the kit
5   comprising one or more of, two or more of and preferably
all of the following primer pairs and accompanying
identification probes.

HPV 16:HPV16.txt 7905 b.p

10

HPV16P2:

p2:116 (20)   GATGCAAGGTCGCATATGAGCCACAGGAGCGACCCAGAAA

16 p1 (no7)

AATTCTAATACGACTCACTATAGGGAGAAGG ATT CCC ATC TCT ATA TAC TA (51
15   baser)

HPV16PO2:
po:230 (20)   TATGACTTTGCTTTTCGGGA
H16e6702po

20

HPV 18:HPV18.txt 7857 b.p

HPV18P2:
25   p2:698 (22)   GATGCAAGGTCGCATATGAGGAAAACGATGAAATAGATGGAG
H18e6702p2
HPV18P4:
p1:817 (20)
AATTCTAATACGACTCACTATAGGGAGAAGGGGTCGTCTGCTGAGCTTTCT
30   H18e6703p1 (Multiplex)

HPV18PO2:
po:752 (21)   GAACCACAACGTCACACAATG
35   H18e6702po

HPV 31:HPV31.txt 7912 b.p

40   HPV31P3:
p2:617 (20)   GATGCAAGGTCGCATATGAGACTGACCTCCACTGTTATGA

H31e6703p2
p1:766 (20)
AATTCTAATACGACTCACTATAGGGAGAAGGTATCTACTTGTGTGCTCTGT
H31e6703p1

5

HPV31PO4:
po:686 (26)   GGACAAGCAGAACCGGACACATCCAA
H31e6704po

10

HPV 33:HPV33.txt 7909 b.p

HPV33P1:
p2:618 (22)   GATGCAAGGTCGCATATGAGTATCCTGAACCAACTGACCTAT
H33e6701p2

15

p1:763 (19)
AATTCTAATACGACTCACTATAGGGAGAAGGTTGACACATAAACGAACTG
H33e6701p1

20   HPV33PO3:
po:699 (23)   GGACAAGCACAACCAGCCACAGC
H33e6703po

As alternative to the probes shown above the kit may
25   optionally include one or more of the following
molecular beacon probes:

Molecular Beacon Probes:

30
H16e6702mb2-FAM   ccagctTATGACTTTGCTTTTCGGGAagctgg

H18e6702mb1-TxR   cgcatgGAACCACAACGTCACACAATGcatgcg

35   H31e6704mb2-FAM   ccgtcgGGACAAGCAGAACCGGACACATCCAAcgacgg

H33e6703mb1-FAM   ccaagcGGACAAGCACAACCAGCCACAGCgcttgg

Preferably the kit of the invention also includes the
40   following primer pair and probe. :

HPV45: HPV45.txt 7858 bp (X74479)

HPV45P1:

p2:430 (21):   GATGCAAGGTCGCATATGAGAACCATTGAACCCAGCAGAAA

5   H45e6701p2

p1:527 (22):

AATTCTAATACGACTCACTATAGGGAGAAGGTCTTTCTTGCCGTGCCTGGTCA

H45e6701p1

10   HPV45PO1:

po:500 (20):   GTACCGAGGGCAGTGTAATA

H45e6701po

The HPV 45 probe above may be replaced by an HPV

15   molecular beacon probe as follows:

H45e6701mb1   cgatcgGTACCGAGGGCAGTGTAATAcgatcg

In addition the kit may include one or more of the

20   following primer pairs and accompanying identification

probes depending on the geographical area of use of the

kit.

HPV52: HPV52.txt 7942 bp (X74481)

25

HPV52P1:

· p2:144 (22):   GATGCAAGGTCGCATATGAGTTGTGTGAGGTGCTGGAAGAAT

H52e6701p2

p1:358 (18):   AATTCTAATACGACTCACTATAGGGAGAAGGCCCTCTCTTCTAATGTTT

30   H52e6701p1

HPV52PO1:

Po:296 (20):   GTGCCTACGCTTTTTATCTA

H52e6701po

35

HPV58 HPV58.txt 7824 bp (D90400)

HPV58P2:

p2:173 (18):   GATGCAAGGTCGCATATGAGTCTGTGCATGAAATCGAA

H58e6702p2

p1:291 (18):

AATTCTAATACGACTCACTATAGGGAGAAGGAGCACACTTTACATACTG

H58e6702p1

HPV58PO2:

po:218 (22):   TTGCAGCGATCTGAGGTATATG

H58e6702po

HPV51 HPV51.txt 7808 bp (M62877)

HPV51PA/P:

p2:655 (23):   GATGCAAGGTCGCATATGAG AGA GGA GGA GGA TGA AGT AGA TA

H51e6702p2

p1:807 (20):   AATTCTAATACGACTCACTATAGGGAGAAGG GCC CATTAA CAT CTG

CTG TA H51e6701p1

HPV51POA:

po:771 (24):   TGG CAG TGG AAA GCA GTG GAG ACA

H51e67o2po

The probes shown above may be replaced in the kit by the
following molecular beacon probes:

H52e6701mb1   cgatcgGTGCCTACGCTTTTTATCTAcgatcg

H58e6702mb1   ccgtcgTTGCAGCGATCTGAGGTATATGcgacgg

H51e6702mb1   cgatcgTGG CAG TGG AAA GCA GTG GAG ACAcgatcg

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CLAIMS

1.   An *in vitro* method of screening human subjects to assess their risk of developing cervical carcinoma which comprises screening the subject for expression of mRNA transcripts from the L1 gene and the E6 gene of human papillomavirus, wherein subjects positive for expression of L1 and/or E6 mRNA are scored as being at risk of developing cervical carcinoma.

2.   An *in vitro* method of screening human subjects to assess their risk of developing cervical carcinoma which comprises screening the subject for expression of mRNA transcripts of the L1 gene of human papillomavirus (HPV) and mRNA transcripts of the E6 gene of HPV, and sorting the subject into one of four categories of risk for development of cervical carcinoma based on expression of L1 and/or E6 mRNA according to the following classification:

Risk category 1: subjects negative for expression of L1 mRNA but positive for expression of E6 mRNA from at least one of HPV types 16, 18, 31, 33, 35, 39, 45, 52, 56, 58, 59, 66 and 68;

Risk category 2: subjects positive for expression of L1 mRNA and positive for expression of E6 mRNA from at least one of HPV types 16, 18, 31, 33, 35, 39, 45, 52, 56, 58, 59, 66 and 68;

Risk category 3: subjects positive for expression of L1 mRNA but negative for expression of E6 mRNA;

Risk category 4: subjects negative for expression of L1 mRNA and negative for expression of E6 mRNA.

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3.   A method according to claim 1 which further comprises screening for expression of p16^{ink4a}, wherein subjects positive for expression of L1 and/or E6 mRNA and positive for expression of p16^{ink4a} are scored as
5   being at risk of developing cervical carcinoma.

4.   An *in vitro* method of screening human subjects for the presence of integrated HPV or a modified episomal HPV genome, which method comprises
10   screening the subject for expression of mRNA transcripts from the L1 gene and the E6 gene of human papillomavirus, wherein subjects negative for expression of L1 mRNA but positive for expression of E6 mRNA are scored as carrying integrated HPV or a
15   modified episomal HPV genome.

5.   An *in vitro* method of screening human subjects for the presence of integrated HPV or a modified episomal HPV genome, which method comprises
20   screening the subject for expression of mRNA transcripts from the E6 gene of human papillomavirus, wherein subjects positive for expression of E6 mRNA are scored as carrying integrated HPV or a modified episomal HPV genome.

25
6.   An *in vitro* method of screening human subjects to assess their risk of developing cervical carcinoma, which method comprises screening the subject for expression of mRNA transcripts of the E6
30   gene of HPV and sorting the subject into one of two categories of risk for development of cervical carcinoma based on expression of E6 mRNA, wherein individuals positive for expression of E6 mRNA are scored as carrying integrated HPV or a modified
35   episomal HPV genome and are therefore classified as high risk for development of cervical carcinoma, whereas individuals negative for expression of E6 mRNA

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are scored as not carrying integrated HPV or a
modified episomal HPV genome and are therefore
classified as no detectable risk for development of
cervical carcinoma.

5

        7.   An *in vitro* method of identifying human
subjects having abnormal cell changes in the cervix,
which method comprises screening the subject for
expression of mRNA transcripts of the E6 gene of HPV,
10   wherein individuals positive for expression of E6 mRNA
are identified as having abnormal cell changes in the
cervix.

        8.   A method according to any one of claims 5 to
15   7 wherein the human subjects are subjects previously
identified as infected with human papillomavirus DNA
in cells of the cervix.

        9.   A method according to any one of claims 5 to
20   7 wherein the human subjects are subjects having a
previous diagnosis ASCUS, CIN 1 lesions or condyloma.

        10.   A method according to any one of claims 4 to
9 wherein individuals positive for expression of E6
25   mRNA from at least one of HPV types 16, 18, 31, 33 or
·45 are scored as carrying integrated HPV.

        11.   A method according to any one of claims 1 to
4 which comprises screening for L1 mRNA expression
30   using a technique which is able to detect L1 mRNA from
substantially all known HPV types.

        12.   A method according to any one of claims 1 to
11 which comprises screening for E6 mRNA expression
35   using a technique which is able to detect E6 mRNA from
at least one cancer-associated HPV type,

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13. A method according to claim 12 which comprises screening for E6 mRNA expression using a technique which is able to detect E6 mRNA from HPV types 16, 18, 31, 33, and preferably 45.

5

14. A method according to any one of claims 1 to 13 wherein screening for L1 and/or E6 mRNA expression is carried using an amplification reaction to amplify of a region of the mRNA, together with real-time

10   detection of the products of the amplification reaction.

15. A method according to claim 14 wherein screening for L1 and/or E6 mRNA expression is carried

15   using real-time NASBA.

16. A kit for use in the detection of mRNA transcripts of the L1 and E6 genes of HPV, the kit comprising at least one primer-pair suitable for use

20   in amplification of a region of L1 transcripts from at least HPV types 16, 18, 31, 33, and preferably 45 and one or more primer-pairs which enable amplification of a region of E6 transcripts from HPV types 16, 18, 31, 33, and preferably 45.

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FIG. 1A.

HPV16-FAM
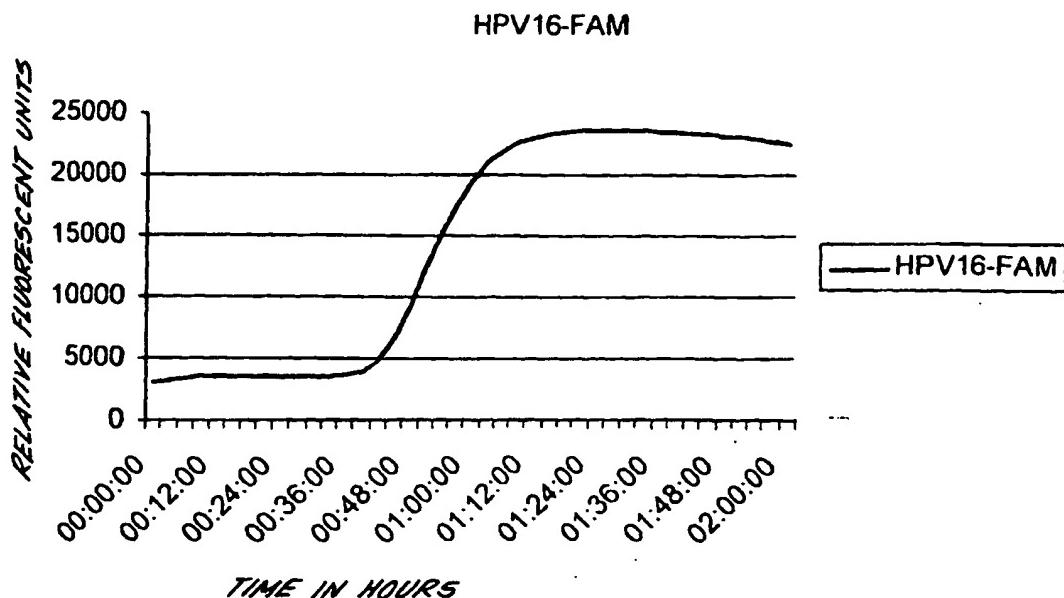
FIG. 1B.
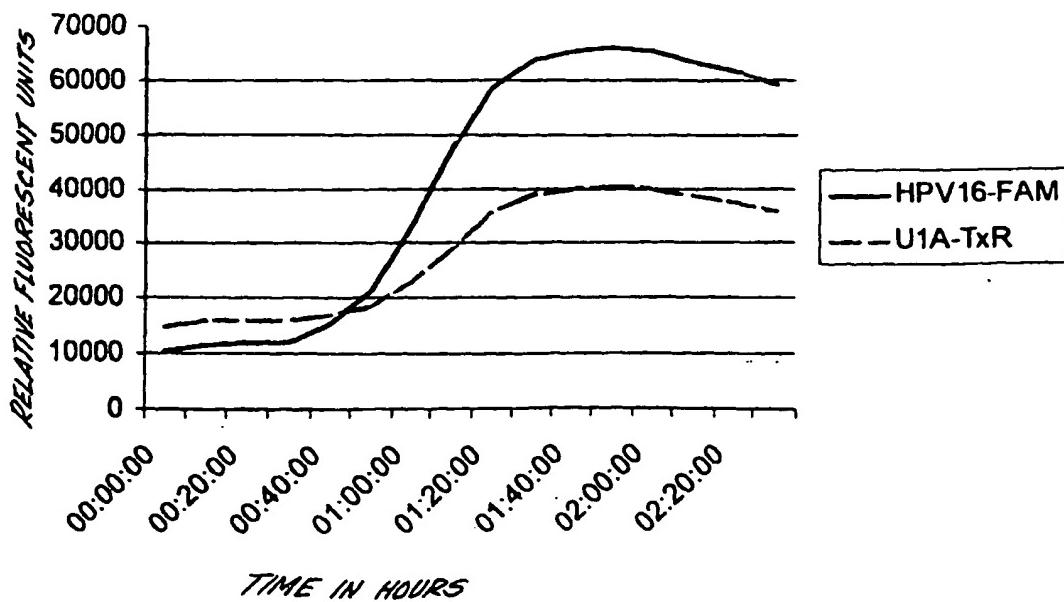
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FIG. 2A.

HPV18-FAM

FIG. 2B.

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FIG. 3A.

HPV31-FAM

FIG. 3B.

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FIG. 4A.

HPV33-FAM

FIG. 4B.

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FIG.5A.

HPV45-FAM

FIG.5B.

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FIG. 6.

◨	PCR Consensus	
☐	PCR Typing	
▨	HPV-Proofer	